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SEQUESTERED ALKALOID DEFENSES IN THE DENDROBATID POISON FROG OOPHAGA PUMILIO PROVIDE VARIABLE PROTECTION FROM MICROBIAL PATHOGENS

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ABSTRACT

Amphibians produce the majority of their defensive chemicals, however alkaloid defenses in poison frogs are sequestered from dietary arthropods. Alkaloids function as a defense against predators, and certain types appear to inhibit microbial growth. However, alkaloid defenses vary considerably among populations of poison frogs, reflecting geographic differences in availability of dietary arthropods. Consequently, environmentally driven differences in poison frog alkaloid defenses may have significant implications regarding their protection against pathogens. While natural alkaloid mixtures in poison frogs have recently been shown to inhibit growth of non-pathogenic microbes, no studies have examined the effectiveness of alkaloids against microbes that infect frogs. Herein, I examined how alkaloid defenses in the strawberry poison frog, *Oophaga pumilio*, affect growth of the known anuran pathogens *Aeromonas hydrophila* and *Klebsiella pneumoniae*. Frogs were collected from five locations throughout Costa Rica that are known to vary in their alkaloid profiles. Alkaloids were isolated from individual skins, and extracts were assayed against both pathogens. Microbe subcultures were inoculated with extracted alkaloids to create dose-response curves. Subsequent spectrophotometry and cell counting assays were used to assess growth inhibition. GC-MS was used to characterize and quantify alkaloids in frog extracts, and my results suggest that variation in alkaloid defenses lead to differences in inhibition of these pathogens. This study provides the first evidence that alkaloid variation in a dendrobatid

poison frog is associated with differences in inhibition of anuran pathogens, and offers further support that alkaloid defenses in poison frogs confer protection against both pathogens and predators.

INTRODUCTION

Predation and disease are important selective pressures that affect individuals, populations, and communities, thereby driving the evolution of highly diversified defensive strategies (Edmunds, 1974). Among these strategies, the use of unpalatable or toxic compounds as a defense mechanism is a common adaptation present in a variety of microorganisms, plants, invertebrates, and vertebrates (reviewed in Wolfe, 2000; Mebs, 2001; Engel *et al*., 2002; Fürstenberg-Hägg *et al*., 2013). Among vertebrates, chemical defenses are widely known for protecting against predators, but in some cases, they also play a critical role in defending against pathogenic microorganisms (Nicolas and Mor, 1995). Amphibians, for example, have characteristically thin, permeable integuments and often inhabit moist environments that favor the growth of bacteria and fungi; as a result, many amphibians have developed defensive systems primarily based on chemicals (Bevins and Zasloff, 1990; Nicolas and Mor, 1995; Conlon, 2011a).

All amphibians have dermal granular glands, which develop during late larval stages and proliferate in size and number throughout the integument after metamorphosis (Vanable, 1964; Toledo and Jared, 1995). Granular glands are syncytial structures enveloped by smooth muscle cells and connected to the epidermis via ducts (Dockray and Hopkins, 1975; Neuwirth *et al*., 1979; Toledo and Jared, 1995). These glands are responsible for releasing skin secretions (i.e., chemicals) intended to defend against predators, parasites, and pathogens (Toledo and Jared, 1995; Rivas *et al*., 2009; Conlon, 2011a, 2011b; Savitzky *et al*., 2012). Accordingly, amphibian granular glands secrete a wide variety of bioactive chemicals in response to stimulation by stress or injury (Toledo and Jared, 1995; Roelants *et al*., 2013), including biogenic amines (Daly *et al*., 1987;

Erspamer, 1994; McClean *et al*., 2002), peptides and proteins (Rollins-Smith, 2005; Conlon, 2011a, 2011b), steroidal bufadienolides (Daly *et al*., 2004; Erspamer, 1994), tetrodotoxin and related analogs (Yotsu *et al*., 1990; Kim *et al*., 2003; Cardall *et al*., 2004; Daly, 2004; Yotsu-Yamashita *et al*., 2004), indolic alkaloids (Daly and Garraffo, 1990; Jeckel *et al*., 2015a), and an array of lipophilic alkaloids (Daly *et al*., 2005; Saporito *et al*., 2012). Amphibians synthesize the majority of these chemical defenses (Daly, 1995; Toledo and Jared, 1995), but some antimicrobial peptides and metabolites are provided by microbial symbionts (Brucker *et al*., 2008; Harris *et al*., 2009; Becker and Harris, 2010; Loudon *et al*., 2014), and lipophilic alkaloids are acquired through diet (Daly *et al*., 1994; Saporito *et al*., 2009). While thousands of unique compounds have been identified from amphibian skin secretions over the past several decades (Erspamer, 1994; Daly, 1995; Daly *et al*., 2005; Xu and Lai, 2015), research has focused largely on synthesized (i.e., antimicrobial peptides) rather than acquired defenses.

Peptides are well-studied and commonly synthesized defensive compounds secreted by the majority of amphibians (Bevins and Zasloff, 1990; Pukala *et al*., 2006). Some peptides play a role in protecting against predators and parasites (Spindel, 2006; König *et al*., 2012), and many are considered key components in the amphibian innate immune system due to their broad-spectrum antimicrobial activities (Nicolas and Mor, 1995; Diamond *et al*., 2009; Conlon, 2011b). Antimicrobial peptides are amphipathic, cationic peptides that are 10-50 amino acid residues in length and express cytolytic activity against pathogenic bacteria, fungi, viruses, and protozoa (Nicolas and Mor, 1995; Conlon, 2011b). Approximately 1,900 antimicrobial peptides, classified into nearly 100 families, have been identified from 178 amphibian species in 28 genera (reviewed in Xu

and Lai, 2015). At the species level, amphibian skin secretions often contain multiple members of a single peptide family (Tennessen and Blouin, 2007); the heterogeneity of molecular structure among peptides within each family is substantial, and this is reflected in the observed variation of antimicrobial activity and microbe specificity between peptides (Tennessen *et al*., 2009; Conlon, 2011a). The diversity of peptides produced by individual amphibian species may confer protection against a wider range of microbes compared to those that produce relatively homogenous mixtures, and interactive effects between peptides may increase antimicrobial efficacy (Nicolas and Mor, 1995; Simmaco *et al*., 1998b; Zasloff, 2002; Rosenfeld *et al*., 2006). Not all amphibians secrete synthesized antimicrobial peptides, but in general, those that do secrete peptides appear to have an advantage in terms of protection from infection (Conlon, 2011b). In contrast, amphibians that rely on external and unpredictable sources for defensive compounds (e.g., lipophilic alkaloids) are only able to sequester what is available to them, which may influence their protection from predators and pathogens (Mina *et al*., 2015). Lipophilic alkaloids represent a suite of acquired chemical defenses that are, in many ways, analogous to the diversity of synthesized peptide defenses present in many amphibians. Moreover, lipophilic alkaloid defenses provide an opportunity to examine the antimicrobial efficacy of sequestered chemical defenses, which has gone largely unstudied.

Poison frogs are a polyphyletic group of brightly-colored anurans that accumulate and secrete lipophilic alkaloids sequestered from their diet as a defense. This globally-distributed assemblage is represented by approximately 150 species from seven lineages in the families Bufonidae (*Melanophryniscus*), Dendrobatidae (alkaloid sequestration

evolved independently in Dendrobatinae, *Ameerega*, and *Epipedobates*), Eleutherodactylidae (*Eleutherodactylus*), Mantellidae (*Mantella*), and Myobatrachidae (*Pseudophryne*) (reviewed in Saporito *et al*., 2012). Experimental evidence of alkaloid sequestration in bufonids (Hantak *et al*., 2013), dendrobatids (Daly *et al*., 1994), mantellids (Daly *et al*., 1997), and myobatrachids (Smith *et al*., 2002) suggests that poison frogs uptake and store an assortment of lipophilic alkaloids (i.e., alkaloid cocktails) obtained from alkaloid-containing dietary arthropods. In particular, mites and ants provide the majority of alkaloids identified in poison frogs (Daly *et al*., 2002; Saporito *et al*., 2004, 2007b, 2009; Takada *et al*., 2005), though certain classes of alkaloids may also be derived from coccinellid beetles (Daloze *et al*., 1995) and siphonotid millipedes (Saporito *et al*., 2003). The lipophilic alkaloids that poison frogs sequester from arthropods are numerous and highly diverse, with over 1,200 unique alkaloids identified and separated into 28 different structural classes (Daly *et al*., 2005; Garraffo *et al*., 2012; Saporito *et al*., 2012; Jeckel *et al*., 2015a). Individual poison frogs accumulate between one and 69 of these distinct alkaloids in varying amounts (Daly *et al*., 2005; Saporito *et al*., 2006, 2007a; Mina *et al*., 2015), although there is no known maximum number that can be accumulated; conversely, dendrobatid poison frogs are not known to synthesize antimicrobial peptides, although trace amounts of the peptide carnosine have been detected in one species of dendrobatid poison frog to date (Daly *et al*., 1984, 1987; Erspamer *et al*., 1986; Prates *et al*., 2012; see Jeckel *et al*., 2015a for review). Just as producing a variety of peptides enhances protection from a wider range of pathogens (Rosenfeld *et al*., 2006; Conlon, 2011b), more diverse alkaloid cocktails may provide protection from a wider range of pathogens, though this has yet to be tested.

Alkaloids in poison frogs appear to function as a defense against predators primarily due to their unpalatability, and in some cases, toxicity (Brodie and Tumbarello, 1978; Stynoski *et al*., 2014; Hantak *et al*., 2016; Murray *et al*., 2016). Additionally, they may confer protection against pathogenic microorganisms. For example, the presence of alkaloids throughout non-integumentary tissue (including muscle and liver tissues, as well as oocytes) in the same relative proportions as in the skin of the bufonid poison frog, *Melanophryniscus simplex,* may indicate that alkaloids play a role in protection from internal infection by non-integumentary parasites (Grant *et al*., 2012). An alkaloid found commonly in some dendrobatid poison frogs, pumiliotoxin **251D**, may also be a highly effective deterrent of a tropical mosquito known to parasitize anurans (Weldon *et al*., 2006). Furthermore, individual synthetic and natural alkaloids inhibit the growth of common microbes, including the bacteria *Bacillus subtilis* (gram-negative) and *Escherichia coli* (gram-positive), and the fungus *Candida albicans* (Macfoy *et al*., 2005). Most recently, natural alkaloid cocktails from geographically separate populations of the dendrobatid poison frog, *Oophaga pumilio*, were found to inhibit growth of those same common microbes to varying degrees, suggesting that alkaloid defenses may vary among populations in their ability to inhibit microbial growth (Mina *et al*., 2015).

The individual alkaloids evaluated for antimicrobial activity by Macfoy *et al*. (2005) were primarily synthetic enantiomers of poison frog alkaloids, although some individual natural alkaloids were included. In general, piperidine, pumiliotoxin, and pyrrolidine alkaloids were the most inhibitory, and individual alkaloids were most effective against *B. subtilis* and *C. albicans*, but rarely effective against *E. coli* (Macfoy *et al*., 2005). Antimicrobial assays testing natural alkaloid cocktails from different

populations of *O. pumilio,* on the other hand, suggest that the bacteria *B. subtilis* and *E. coli* are more effectively inhibited by alkaloid defenses than the fungus *C. albicans* (Mina *et al*., 2015). While this may indicate that natural alkaloid defenses of *O. pumilio* are less effective at inhibiting fungal microbes, the ecological relevance of this is unknown as the microbes tested were not frog pathogens.

Alkaloid defenses vary considerably among dendrobatid poison frog species, populations, and individuals within populations, but geographically close populations generally have more similar alkaloid defenses than geographically distant populations (Myers and Daly, 1976; Daly *et al*., 1987, 2008a; Saporito *et al*., 2006, 2007a). Although certain factors such as species, sex, age, and life stage are important determinants of alkaloid variation (Saporito *et al*., 2010a, 2010b, 2012; Stynoski *et al*., 2014; Jeckel *et al*., 2015b), geographic differences in alkaloid defenses seem to be a direct result of variation in the local availability of dietary arthropods (Saporito et al., 2007a). Given that antimicrobial activity differs among individual alkaloids (Macfoy *et al*., 2005) and natural alkaloid cocktails (Mina *et al*., 2015), environmentally-driven differences in alkaloid defenses may influence frog protection against natural pathogens at the population level. For example, differential resistance among poison frog populations to infectious pathogens may correlate with survival probability during outbreaks of emerging infectious diseases. Moreover, the combination of geographic and temporal differences in pathogen prevalence and variable pathogen protection among poison frog populations may influence species distributions and persistence in the wild. Poison frogs in captivity may be even more vulnerable since alkaloids are acquired exclusively through a specialized diet of alkaloid-containing arthropods (Daly *et al*., 1994; Saporito

et al., 2009); frogs entirely lack defenses if raised in captivity (Daly *et al*., 1992, 1997; Hantak *et al*., 2013), while frogs collected from nature are left with only the alkaloids contained in their granular glands at the time of collection, which can persist for over six years in captivity (Myers *et al*., 1978; St. Claire *et al*., 2005). As a result of global amphibian population declines, *ex situ* captive breeding programs with the goal of facilitating successful reintroductions to the wild have been initiated for a variety of endangered amphibians (for review, see Griffiths and Pavajeau, 2008; Harding *et al*., 2015), some involving endangered poison frogs (Hunter et al., 2010b; McFadden et al., 2013; Edmonds et al., 2015). If poison frogs with reduced or absent alkaloid defenses are truly more vulnerable to infectious pathogens, captive breeding programs for poison frogs may greatly benefit from research that informs management and reintroduction strategies that are more likely to succeed, which is crucial in light of contemporary biodiversity loss and the global amphibian crisis.

Population declines and species extinctions resulting in losses of biodiversity are occurring at rates that are orders of magnitude higher than the average rate inferred from the fossil record (Myers, 1993; Singh, 2002). Of all vertebrate taxa, amphibians are experiencing the greatest global biodiversity decline (Stuart *et al*., 2004), and although there are many contributing factors (Blaustein and Kiesecker, 2002), infectious disease appears to be a major cause (Daszak *et al*., 1999). Numerous pathogenic microbes infect amphibians, including opportunistic gram-negative bacteria, the chytrid fungus *Batrachochytrium dendrobatidis* (*Bd*), mycobacteria, ranaviruses, water molds, trematodes, and other various parasites (Taylor *et al*., 2001; Green *et al*., 2002; Densmore and Green, 2007; Schadich and Cole, 2010; Haridy *et al*., 2014). While the majority of

research on amphibian disease in relation to population declines to date has focused on *Bd*, other pathogens are involved as well and have been comparatively understudied (see Green *et al*., 2002; Prada-Salcedo *et al*., 2011). For example, *Aeromonas hydrophila*, ranaviruses, mycobacteria, and water molds are reported to co-occur with chytrid infections in captive and wild populations of frogs (Miller *et al*., 2008; Hill *et al*., 2010; Prada-Salcedo *et al*., 2011; Whitfield *et al*., 2013); therefore, understanding how amphibians deal with these other pathogens alone or in co-infection with *Bd* will provide valuable insight into the role of these microbes in amphibian declines.

Opportunistic gram-negative bacteria can cause bacterial dermatosepticemia, a fatal systemic infectious disease in frogs that is implicated in population declines and extirpations in the wild (Hunsaker and Potter, 1960; Nyman, 1986; Bradford, 1991; Sherman and Morton, 1993) and mass mortalities in captivity (Olson *et al*., 1992; Pearson, 1998; Green *et al*., 1999; Mauel *et al*., 2002). Although a variety of gram-negative bacteria can cause bacterial dermatosepticemia, certain bacteria seem to be implicated in the majority of cases (e.g., *A. hydrophila*). Some bacteria also appear to be more virulent than others, dependent on frog species as well as a variety of environmental, stress, dietary, health, and immune status factors (Schadich and Cole, 2010). For instance, Schadich and Cole (2010) isolated *A. hydrophila*, *Klebsiella pneumoniae*, and *Proteus mirabilis* from brown tree frogs (*Litoria ewingii*) during an outbreak of bacterial dermatosepticemia in a New Zealand forest; through exposure of healthy frogs to the three pathogen isolates, *K. pneumoniae* was determined to be the most pathogenic, despite *A. hydrophila* typically being the reported causative agent in bacterial dermatosepticemia epidemics. Moreover, *L. ewingii* lacks skin peptide defenses

(Schadich, 2009), which supports the idea that chemical defenses in frogs are important for protection from these pathogens, considering how susceptible to infection *L. ewingii* appears to be. For instance, resistance of *Litoria raniformis* to *K. pneumoniae* is related to the antimicrobial activity of its peptide defenses (Schadich *et al*., 2009), and resistance of certain Australian and Central American frogs to *Bd* is correlated with the antimicrobial activity of peptides (Woodhams *et al*., 2006a, 2006b, 2007a). Although antimicrobial peptide defenses are thought to have evolved as a secondary, broad-spectrum immune defense (Nicolas and Mor, 1995; Roelants *et al*., 2013) and are effective in that role (Simmaco *et al*., 1998b; Zasloff, 2002; Rollins-Smith, 2005), there is a fundamental gap in our understanding of the role that sequestered alkaloid defenses serve in defending poison frogs from infection.

Research on the antimicrobial activity of alkaloid defenses in frogs (Macfoy *et al*., 2005; Mina *et al*., 2015) has focused on the strawberry poison frog, *O. pumilio*, a terrestrial dendrobatid frog whose alkaloid defenses are relatively well-characterized and vary considerably with geographic location at both small and large spatial scales (Saporito *et al*., 2006, 2007a, 2010a). Moreover, although *O. pumilio* is not at risk for decline due to infectious pathogens, individual and co-occurring infections by pathogenic microbes are prevalent in certain populations (Whitfield *et al*., 2013). The substantial variability in *O. pumilio* alkaloid defenses, in addition to an abundance of previously-identified alkaloids, makes *O. pumilio* an ideal study organism in which to examine ecologically relevant antimicrobial activity of sequestered alkaloid defenses. The aim of the present study is to examine the extent to which the variable alkaloid defenses in a dendrobatid poison frog, *Oophaga pumilio*, inhibit the growth of the anuran pathogens *A.*

hydrophila and *K. pneumoniae*. Comparing highly variable alkaloid defenses from individual *O. pumilio* with corresponding microbial inhibition allows direct evaluation of the extent to which inhibition is driven by alkaloid defenses. On the basis of previous studies, I hypothesized that population-level variation in alkaloid defenses will differentially inhibit microbial growth, dependent upon the quantity and types of alkaloids present. Specifically, populations with greater quantities of alkaloids and more diverse alkaloid cocktails are predicted to inhibit microbial growth most effectively.

METHODS

Study Sites and Frog Collections

Adult frogs were collected from five sites located in lowland tropical rainforest throughout eastern Costa Rica and were selected based on known differences in the alkaloid defenses (i.e., number and quantity of unique alkaloids and alkaloid structural classes) of *O. pumilio* from each site (**Fig. 1**; alkaloid data from Saporito *et al.*, 2007a). La Selva Biological Station (10°26' N, 83°59' W) and Finca los Nacientes (10°21’53” N, 84°8’6” W) are located in Heredia Province. La Selva is a private biological reserve managed by the Organization for Tropical Studies, and is dominated by primary forest that has experienced minimal human impact since the European colonization of the Americas (Whitfield *et al*., 2012). The other three sites, Tortuguero (10°35’14” N, 83°31’34” W), Río Palmas (10°10’16” N, 83°36’26” W), and Gandoca (9°35’03” N, 82°37’13” W), are located throughout Limón Province, which spans the entire eastern coast of Costa Rica.

In order to acquire natural frog alkaloids for microbial growth assays, 14 adult *O. pumilio* were collected from each of the five geographically isolated sites throughout Costa Rica (n = 14 frogs per population, N = 70 total). This sample size allowed for variation in alkaloid defenses to be examined within and among populations and between sexes, and was based on the permission allowed by the government of Costa Rica. At each site, a single 45 m × 45 m collection plot was established following the methods of Saporito *et al*. (2006, 2007a, 2010). Selection of a single collection location at each site minimized the variation in alkaloid samples obtained within populations since availability of dietary arthropods varies geographically and since *O. pumilio* has a patchy distribution throughout its range. Frogs within the delineated plot were collected using hand nets and then sexed based on gular skin patches (Donnelly, 1989) until an even sex ratio of seven males and seven females were acquired (except at Río Palmas and Finca Los Nacientes, where eight males and six females were collected due to difficulty locating females).

Collected frogs were weighed to the nearest 0.01 g using a Pesola PPS200 digital pocket scale, measured for snout-to-vent length (SVL) to the nearest 0.1 mm using Control Company Traceable digital calipers, and then euthanized by freezing. Frog skins were collected and stored individually in 4 mL of 100% methanol inside sealed glass vials with Teflon-lined caps. Each vial was labeled with a unique identifier, allowing for extracts to be tracked throughout experimentation and matched to their frog's collection location, sex, weight, and SVL. All methods were approved by John Carroll University IACUC protocol #1101 and collection of *O. pumilio* was approved by CITES collection permit 2015-CR1420/SJ(#S1487).

Alkaloid Fractionations

Alkaloids from each of the 70 frog skins were extracted into individual 100 µL fractions using an acid-base extraction technique that isolates lipophilic alkaloids from other compounds such as fatty acids and carotenoids (original methods outlined in Saporito *et al*., 2010). For each skin sample, 1 mL of the original 4 mL methanol extract was transferred to a 10 mL conical vial and acidified with 50 µL hydrochloric acid (1 N) to solubilize the alkaloids in water. This solution was then evaporated to a volume of 100 µL using nitrogen gas, followed by dilution with 200 µL deionized water. Four subsequent extractions with 300 µL hexane removed the majority of non-alkaloid lipophilic compounds from the samples. The organic hexane layer was disposed of, and the remaining aqueous solution containing alkaloids was basified with eight drops of sodium bicarbonate such that alkaloids became soluble in ethyl acetate. Once basicity was verified with pH paper, three subsequent extractions with 300 µL ethyl acetate then separated alkaloids from residual non-alkaloid compounds in the aqueous solution. Each time, the ethyl acetate layer was pipetted into a tube with anhydrous sodium sulfate to remove remaining trace amounts of water. The total 900 µL of ethyl acetate containing alkaloids was transferred to a clean tube and evaporated until dryness with nitrogen gas. Alkaloid fractions were immediately resuspended in 100 µL methanol as soon as all liquid evaporated, and this 100 µL fraction was transferred to a microvial with a Teflon-lined cap for use in microbial growth inhibition assays. Alkaloid extractions were conducted twice for each frog skin (n = 140 individual fractionations), resulting in a final volume of 200 µL alkaloids available for use in microbial growth inhibition assays. The two 100 µL alkaloid fractions from each frog were combined to eliminate any variation

between fractions from the same frog skin, and then the alkaloid fractions from all frogs were used in microbial growth inhibition assays (see below). For GC-MS analyses of alkaloid fractions, this procedure was carried out one additional time for each skin sample (n = 70 individual fractionations), but with the addition of 100 µL nicotine immediately after the acidification step as an internal standard.

Culture and Maintenance of Microbes

All procedures involving microbial cultures and growth assays were performed in a HEPA filtered laminar flow hood (SterilGARD Hood, The Baker Company, Sanford, ME) with barrier pipet tips (AvantGuard, MIDSCI, St. Louis, MO), and all media was sterilized before use by autoclaving at 121°C for 30 minutes. *Aeromonas hydrophila* (ATCC® 35654™) and *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC® 13883™) stock cultures were each grown in 50 mL polypropylene conical tubes (SpinMax, Crystalgen, Commack, NY) containing 30 mL tryptic soy broth (TSB) (Remel, Thermo Scientific, Lenexa, KS), and incubated for 24 hours at 30°C (*A. hydrophila*) or 37°C (*K. pneumoniae*) in a shaking incubator (New Brunswick Scientific 12400 Incubator Shaker) set to 100 revolutions per minute (rpm). All procedures involving incubation and/or shaking were conducted under these temperature and agitation conditions. Twelve 1 mL stock cultures per microbe were cryopreserved at -80°C (see **Cryopreservation of Microbes** in **APPENDIX**). Each week, a 10 µL loop from a new thawed stock culture was streaked on a tryptic soy agar (TSA) (Teknova, Hollister, CA) culture plate and incubated until single colonies formed (typically 8 hours). This ensured viability of single colonies that were used to grow cultures for microbial growth inhibition assays.

Microbial Growth Inhibition Assays

Optical Density Assays

Optical density (OD) assays were adapted from an assay described by Mina *et al*. (2015). To begin each OD assay (n = 2 per population, 10 per microbe, 20 total), a 30 mL TSB culture was inoculated with a single pure colony from a streak plate and incubated at 30°C (*A. hydrophila*) or 37°C (*K. pneumoniae*) for 18 hours. Based on pilot growth experiments (see **Pilot Experiments** in **APPENDIX**), these cultures were diluted with TSB to a ratio of 1:10 (*A. hydrophila*) or 1:5 (*K. pneumoniae*) immediately prior to experimentation, allowing all assayed subcultures to proceed through the logarithmic (log) growth phase and into the stationary growth phase within 24 hours. Diluted cultures were vortexed (Vortex Genie 2, VWR, Radnor, PA) for 5 seconds and poured into a plastic trough (VWR, Radnor, PA), and then equal 200 µL volumes (subcultures) of the microbial suspension were immediately aliquotted into 80 wells of a 96-well microplate (Falcon, Corning, Tewksbury, MA) using a multichannel pipettor (20 – 200 µL, BioPette Plus, Labnet International, Edison, NJ).

Column 1 on the microplate represented an untreated control, columns 2 – 5 and 7 – 9 each represented one of seven individual frogs in the assay (inoculation with alkaloid extracts from corresponding frogs), and columns 6 and 10 represented vehicle controls. Methanol alone served as the vehicle control because alkaloid extracts were dissolved in methanol. Excluding column 1, each of the 72 inoculated subcultures per assay were inoculated with either 2.4 (rows A – D) or 4.8 µL (rows E – H) of alkaloid extract or pure methanol. This resulted in a total of four replicates (subsamples) per volume under each treatment (alkaloid or methanol inoculation), except for the untreated control that had

eight replicates in each assay. Since the addition of methanol resulted in consistent growth inhibition, any subsamples that grew an abnormal amount (> 20%) more than untreated controls were considered contaminated and were therefore excluded from analyses. Treatment volumes were selected based on pilot experiments which indicated that 9.6 µL of methanol in 200 µL of *A. hydrophila* was highly inhibitory and would likely obscure any inhibition due to natural alkaloid extracts; while treatments with 2.4 µL and 4.8 µL of methanol were inhibitory compared to negative controls, differences between methanol and alkaloid treatments were still apparent at these volumes (see **Pilot Experiments** in **APPENDIX**). Moreover, the treatment volumes are ecologically relevant for what microbes may be exposed to on frog skin, representing 0.6% and 1.2%, respectively, of the total quantity of alkaloids present in individual frog skins (based on calculations from Gronemeyer, 2013).

Immediately following inoculation, the microplate was placed in a Fisher Scientific Multiskan FC microplate reader inside an incubator (Forma Reach-In Incubator, Thermo Fisher Scientific, Marietta, OH) at the microbe's corresponding growth temperature. An initial measurement of each subculture's OD at 620 nm (OD_{620}) was taken prior to incubation to ensure that subcultures in each assay started off at an OD similar to those in pilot experiments (± 0.02 OD_{620} to account for random variation), suggesting that they would be in mid-logarithmic (mid-log) growth phase. When initial OD was verified, a 24.5-hour program that recorded OD_{620} of each subculture at 15-minute intervals after shaking the plate at medium speed for five seconds was initiated to create 24-hour growth curves for each treatment (see **Appendices 11-30** for growth curves). The final OD_{620} readings of all four subsamples per sample were averaged for

use in statistical analyses of OD assays (except in six instances for *A. hydrophila* and one instance for *K. pneumoniae*, when one subsample in each instance was removed due to abnormal growth in 2.4 µL dose treatments). To equalize sample sizes among treatments, fourteen of the twenty methanol control samples for each microbe were randomly selected for use in statistical analyses.

Colony-forming unit assays

Colony-forming unit (CFU) assays were conducted to assess the viability of subcultures that were exposed to natural alkaloid extracts and methanol treatments for 24 hours. Immediately following the final measurement of OD assays, three randomly-selected subsamples from each treatment (e.g., four wells inoculated with alkaloid extracts) were used in CFU assays. Since some subsamples grew abnormally in pilot experiments (but never more than one of four subsamples), three of four subsamples were randomly selected for the final analysis. This approach also ensured equal sample sizes across treatments.

To quantify viable bacteria in each treatment's subsamples, each of the three selected subsamples were serially-diluted in tenfold increments up to 10^{-7} using microplates. Ten microliters of each serial dilution were then plated on TSA in square 6 × 6 gridded petri dishes (Fisherbrand, Thermo Fisher Scientific, Waltham, MA) and incubated for seven hours or until single colonies (i.e., colony-forming units) formed. Once CFUs were visible, the number of CFUs at each dilution (10^{-1} – 10^{-7}) for each subsample was recorded. When CFU counts exceeded 30, colonies tended to grow into one another and it became difficult to accurately quantify CFUs. Therefore, the serial

dilution that grew CFUs nearest to 30 in number, while still being equal to or below 30, was used to calculate the original cell density (OCD; CFU/mL; equation below) of each subsample. The average of each treatment's three subsample OCD values was used in statistical analyses.

$$\text{Original cell density (CFU/mL)} = \frac{\text{(Colony-forming units)}}{\text{(Dilution factor)} \times \text{(Volume of aliquot in mL)}}$$

In the OCD equation, dilution factor refers to the magnitude of dilution of the quantified aliquot (e.g., 10^{-7}), and volume of aliquot in mL refers to the volume of each subsample's dilutions that were plated (10 µL in this case, or 0.01 mL).

Alkaloid Analysis

To determine which unique alkaloids were present in extracts, and in what quantities (µg), samples of individual frog extracts containing a nicotine standard (1 µg/10 µL; (−)-nicotine ≥99%, Sigma-Aldrich) were analyzed using gas chromatography-mass spectrometry (GC-MS). For each alkaloid extract, multiple 1 µL aliquots were analyzed with a Varian Saturn 2100T ion trap MS instrument coupled to a Varian 3900 GC (using a 30 m x 0.25 mm i.d. Varian Factor Four VG-5ms fused silica column). GC-MS analysis was carried out using a temperature program progressing from 100°C to 280°C (increasing at a rate of 10°C/min) and with helium as the carrier gas (flow rate of 1 mL/min). Both electron impact (EI) MS and chemical ionization (CI) MS were used to analyze samples of alkaloid fractions, with methanol as the CI reagent. In order to more accurately estimate the quantity of alkaloids present in each frog's alkaloid extract, EI-MS was conducted in triplicate for each frog, while CI-MS was performed once for each

frog. In total, four 1 µL samples from each of 70 frogs' alkaloid extract were analyzed (n = 280 GC-MS runs).

Unique alkaloids were identified from GC-MS output of alkaloid extracts by comparing the GC retention times and mass spectra of individual peaks with alkaloids previously identified and reported in poison frogs (Daly *et al*., 2005, 2007, 2008a, 2008b, 2009; Saporito *et al*., 2007a; Garraffo *et al*., 2012; Grant *et al*., 2012; Fitch *et al*., 2013; Andriamaharavo *et al*., 2015). The quantity of unique alkaloids present in each frog was determined using, from each of the three EI runs per frog extract, the ratio of the average area under each alkaloid's peak and the nicotine standard using Varian MS Workstation v.6.9 SPI. Alkaloids that had not yet been reported in the literature (i.e., new alkaloids) were characterized and named if they were present in three or more frogs in quantities greater than 5 µg total.

Statistical Analysis

Since alkaloid composition is a collective measure of the number, type, and quantity of alkaloids within individual frog skins, multidimensional scaling (MDS) was used to visualize differences in alkaloid composition among populations. Analysis of similarity (ANOSIM) was used to detect statistical differences in alkaloid composition among these populations. Multivariate analyses were based on Bray-Curtis similarity matrices and performed using PRIMER-E version 5.

A nested one-way analysis of variance (ANOVA) was used to examine variation of growth inhibition within individual frog alkaloid treatments in both OD and CFU assays for each microbe. In all following analyses, averages of the subsamples were used

for each sample within each treatment. A two-tailed, unpaired, independent samples *t*-test was used to determine the presence of a dose-response relationship between 2.4 and 4.8 µL doses in both OD and CFU assays for each microbe. The effects of alkaloids on microbial growth were assessed using one-way ANOVA; final optical densities (growth inhibition) and CFU/mL after treatment (remaining viable cells) of frog alkaloid and methanol treatments in the 4.8 µL treatment were compared among populations and for each pathogen. Multiple comparisons (Tukey's HSD) were used to identify differences in microbial growth inhibition of frog alkaloids from each population compared to methanol treatments. To determine the effects of frog population and sex on growth inhibition, two-way ANOVA was conducted for each assay with microbe. Linear regression analyses were performed to assess relationships between alkaloid composition (diversity and quantity) and growth inhibition (OD and CFU assays) for each microbe, and to test the relationship between OD and CFU assays for each microbe. All parametric statistical analyses were performed using SPSS version 14.0 and GraphPad Prism version 6.05.

RESULTS

Alkaloid Analysis

GC-MS analysis of 70 *Oophaga pumilio* from five Costa Rican populations (**Fig. 1**) resulted in the identification of 353 unique alkaloids (including isomers) represented by 19 different structural classes (see **Table 1** for the most common alkaloids identified in each population and **Table 2** for all identified alkaloids). The majority of these alkaloids are derived from mites and ants. In total, 27 new alkaloids in 7 different structural classes were identified (**Table 3**). Alkaloid composition of *O. pumilio* differed significantly

among the five populations (Global R = 0.91; p = 0.001; **Fig. 2a**), but not between sexes (Global R = -0.03; p = 0.966). Frogs from Finca los Nacientes contained the largest number and greatest quantity of alkaloids, while frogs from Gandoca contained the lowest number of alkaloids and contained similarly low quantities of alkaloids as frogs from La Selva (see **Table 4** for details on alkaloid composition by population and sex). In general, frogs from Tortuguero and Finca los Nacientes contained about three times as much alkaloid (in terms of quantity, µg per frog skin) as frogs from La Selva, Río Palmas, and Gandoca (**Fig. 2b**).

Alkaloid quantity and diversity are also strongly correlated ($F_{1,68}$ = 49.47, p < 0.0001; R^2 = 0.421; **Fig. 3**), although the relationship appears to be exponential rather than linear. In general, frogs containing higher quantities of alkaloids also possessed more diverse alkaloid cocktails, but fewer new types of alkaloids were represented with increasing alkaloid quantities.

Dose-Response Relationship between 2.4 µL and 4.8 µL Treatments

Exposure of both *A. hydrophila* and *K. pneumoniae* to increased doses of alkaloid treatments indicates that there is a clear dose-response relationship between treatments and microbial growth inhibition. With both *A. hydrophila* and *K. pneumoniae*, respectively, 4.8 µL treatments resulted in consistently lower final optical density readings (t_{138} = 13.19, p < 0.0001; t_{138} = 10.15, p < 0.0001) and reduced viable cell counts (t_{138} = 8.47, p < 0.0001; t_{138} = 6.65, p < 0.0001) in comparison to 2.4 µL treatments. In accordance with the dose-response relationship, differences in microbial growth inhibition between control and alkaloid treatments were more evident in 4.8 µL

treatments; therefore, the results for all following assays and comparisons are reported for 4.8 µL treatments only (**Figs. 4 – 8**).

Aeromonas hydrophila Optical Density Assays

Mean optical density of *A. hydrophila* treated with alkaloid cocktails from the five locations of *O. pumilio* differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 13.04$; $p < 0.0005$; **Fig. 4a**); however, mean optical density of *A. hydrophila* treated with alkaloids from individual frogs did not differ within samples ($F_{18,312} = 1.09$; $p = 0.3600$). The mean optical density of *A. hydrophila* treated with alkaloids from Tortuguero, La Selva, and Finca los Nacientes frogs was significantly less than the methanol control, and the mean optical density of *A. hydrophila* treated with alkaloids from Río Palmas and Gandoca frogs was not significantly less than the methanol control. There was no interaction between the effects of frog population and sex for optical density assays with *A. hydrophila* ($F_{4,60} = 0.92$, $p = 0.4590$).

Aeromonas hydrophila Colony-Forming Unit Assays

Mean viable cell density of *A. hydrophila* treated with alkaloid cocktails differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 15.89$; $p < 0.0005$; **Fig. 4b**); however, mean viable cell density of *A. hydrophila* treated with alkaloids from individual frogs did not differ within samples ($F_{12,234} = 0.90$; $p = 0.5450$). The viable cells recovered from *A. hydrophila* treated with alkaloids from all populations was significantly less than the methanol control. There was a statistically significant interaction between frog population and sex in colony-forming unit assays

with *A. hydrophila* ($F_{4,60} = 3.79$, $p = 0.0080$), although there was no difference in original cell density between sexes after treatment with alkaloid cocktails from frogs ($F_{1,60} = 0.99$, $p = 0.3240$); in other words, the more inhibitory sex varied among populations.

Klebsiella pneumoniae Optical Density Assays

Mean optical density of *K. pneumoniae* treated with alkaloid cocktails from the five locations of *O. pumilio* differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 12.67$; $p < 0.0005$; **Fig. 5a**); however, mean optical density of *K. pneumoniae* treated with alkaloids from individual frogs differed within samples ($F_{18,312} = 2.93$; $p < 0.0005$). The mean optical density of *K. pneumoniae* treated with alkaloids from Tortuguero, Finca los Nacientes, and Gandoca frogs was significantly less than the methanol control, and the mean optical density of *K. pneumoniae* treated with alkaloids from La Selva and Río Palmas frogs was not significantly less than the methanol control. There was no interaction between the effects of frog population and sex for optical density assays with *K. pneumoniae* ($F_{4,60} = 1.99$, $p = 0.1070$).

Klebsiella pneumoniae Colony-Forming Unit Assays

Mean viable cell density of *K. pneumoniae* treated with alkaloid cocktails differed significantly among the five populations of *O. pumilio* and methanol controls ($F_{5,78} = 26.41$; $p < 0.0005$; **Fig. 5b**); however, mean viable cell density of *K. pneumoniae* treated with alkaloids from individual frogs did not differ within samples ($F_{12,234} = 0.52$; $p = 0.8990$). The viable cell density of *K. pneumoniae* treated with alkaloids from all populations was significantly less than the methanol control. There was a statistically

significant interaction between frog population and sex in colony-forming unit assays with *K. pneumoniae* ($F_{4,60}$ = 2.81, p = 0.0330), although there was no difference in original cell density between sexes after treatment with alkaloid cocktails from frogs ($F_{1,60}$ = 0.14, p = 0.7060); similarly to colony-forming unit assays with *A. hydrophila*, the more inhibitory sex varied among populations.

Relationships between Alkaloid Composition and Growth Inhibition

Alkaloid-based growth inhibition of *A. hydrophila* was correlated with both alkaloid diversity and composition of *O. pumilio* (**Fig. 6**). Alkaloid diversity was a moderate predictor of growth inhibition by proxy of both OD ($F_{1,68}$ = 48.60, p < 0.0001, R^2 = 0.417; **Fig. 6a**) and CFU assays ($F_{1,68}$ = 17.92, p < 0.0001, R^2 = 0.209; **Fig. 6b**). Alkaloid quantity was also a moderate predictor of growth inhibition by proxy of both OD ($F_{1,68}$ = 53.46, p < 0.0001, R^2 = 0.440; **Fig. 6c**) and CFU assays ($F_{1,68}$ = 13.79, p = 0.0004, R^2 = 0.169; **Fig. 6d**).

Alkaloid quantity, but not diversity, was a better predictor of growth inhibition of *K. pneumoniae* compared to *A. hydrophila* (**Figs. 6 and 7**). Alkaloid diversity was a moderately weak predictor of both OD ($F_{1,68}$ = 19.05, p < 0.0001, R^2 = 0.219; **Fig. 7a**) and CFU assays ($F_{1,68}$ = 34.22, p < 0.0001, R^2 = 0.335; **Fig. 7b**). However, alkaloid quantity was a comparatively stronger predictor of both OD ($F_{1,68}$ = 63.35, p < 0.0001, R^2 = 0.482; **Fig. 7c**) and CFU assays ($F_{1,68}$ = 68.73, p < 0.0001, R^2 = 0.503; **Fig. 7d**) for *K. pneumoniae*.

Relationship between Optical Density and Colony-Forming Unit Assays

Optical density assays and colony-forming unit assays were strongly correlated for both *A. hydrophila* ($F_{1,68} = 80.88$, $p < 0.0001$, $R^2 = 0.543$; **Fig. 8a**) and *K. pneumoniae* ($F_{1,68} = 85.52$, $p < 0.0001$, $R^2 = 0.557$; **Fig. 8b**). The patterns in growth inhibition among *O. pumilio* populations were identical between the two assays for both microbes (**Figs. 4 and 5**).

DISCUSSION

Alkaloid-based chemical defenses in poison frogs are unpalatable and/or toxic to a variety of invertebrates and vertebrates, and function accordingly as a defense against predators (Brodie and Tumbarello, 1978; Fritz *et al*., 1981; Szelistowski, 1985; Weldon *et al*., 2013; Stynoski *et al*., 2014; Hantak *et al*., 2016; Hovey *et al*., 2016; Murray *et al*., 2016). Individual alkaloids and natural alkaloid cocktails from the dendrobatid poison frog *O. pumilio* have also recently been shown to inhibit the growth of microbes that are generally considered non-pathogenic (Macfoy *et al*., 2005; Mina *et al*., 2015), suggesting that alkaloids may provide a dual defense against both predators and microbes. The present study supports this hypothesis and demonstrates that sequestered alkaloid defenses from various populations of *O. pumilio* inhibit the growth of microbes that infect anurans and contribute to substantial mortality in captivity and in the wild (i.e., pathogenic microbes); however, populations of *O. pumilio* also appear to vary in their ability to inhibit these pathogens.

Alkaloid defenses from each population of *O. pumilio* in the present study effectively inhibited growth of the opportunistic, gram-negative bacteria *A. hydrophila* and *K. pneumoniae*. Poison frogs accumulate unique suites of alkaloid defenses gradually over the course of their lifetime as they consume alkaloid-containing arthropods (Saporito *et al*., 2009). In the present study, the composition of alkaloid defenses (i.e., quantity and diversity) differed significantly among populations (**Fig. 2**; **Table 1**), reflecting disparities in the type and availability of dietary arthropods at each location (Saporito *et al*., 2007a, 2009, 2012). Similarly, the extent of growth inhibition of both *A. hydrophila* and *K. pneumoniae* differed significantly among populations (**Figs. 4 and 5**), most likely as a direct result of each population's distinct alkaloid composition.

The two components that determine alkaloid composition, alkaloid quantity and alkaloid diversity, are strongly correlated in the studied populations of *O. pumilio* (**Fig. 3**). As the quantity of alkaloids in individual frogs increased, so did the number of unique alkaloids acquired. Likewise, alkaloid cocktails in the present study inhibited the growth of *A. hydrophila* and *K. pneumoniae* more effectively as alkaloid quantity and alkaloid diversity increased (**Figs. 6 and 7**). Accordingly, alkaloid cocktails from Finca los Nacientes and Tortuguero frogs inhibited growth of *A. hydrophila* and *K. pneumoniae* most effectively of all populations in both assays (**Figs. 4 and 5**). Finca los Nacientes frogs were protected by alkaloid defenses that were, on average, nearly threefold greater in quantity and 40 – 100% more diverse than those La Selva, Río Palmas, and Gandoca frogs. Alkaloid cocktails from Tortuguero frogs were comparable in quantity to alkaloids from Finca los Nacientes frogs, but were instead similar in diversity to alkaloids from La Selva and Río Palmas frogs and higher in diversity than Gandoca (**Table 4**).

Additionally, the predominant alkaloids present in Finca los Nacientes frog alkaloid cocktails (comprising about half of the total quantity) included 5,8-disubstitited indolizidines, decahydroquinolines, and histrionicotoxins, whereas the most abundant alkaloids in Tortuguero frogs (over 60% of the total quantity) were 5,8-disubstituted indolizidines and 5,6,8-trisubstituted indolizidines (**Table 1**). On the basis of these findings, larger quantities of more diverse alkaloids provide enhanced antimicrobial protection, but 5,8-disubstituted indolizidines, 5,6,8-trisubstituted indolizidines, decahydroquinolines, and histrionicotoxins may also be particularly inhibitory alkaloid classes.

All populations of *O. pumilio* in the present study contained disproportionately high quantities and numbers of mite-derived, branched-chain alkaloids, such as 5,8-disubstituted indolizidines and 5,6,8-trisubstituted indolizidines (**Tables 1 and 2**), which is typical of Costa Rican *O. pumilio* (Saporito *et al*., 2007b, 2009, 2011, 2015). However, the alkaloid cocktails from the same populations also varied widely in inhibitory efficacy despite sharing large proportions of these common mite-derived alkaloids (**Figs. 4 and 5**). The large quantities of these common alkaloids present in the more inhibitory Finca los Nacientes and Tortuguero frogs suggest that these alkaloids may become particularly inhibitory above a certain quantity, which could explain the comparatively reduced inhibition by other populations that share these same alkaloids, but in lower quantities. Alternatively, Finca los Nacientes frogs also contained distinctly high quantities of ant-derived decahydroquinoline and histrionicotoxin alkaloids when compared to other populations (**Table 1**), which may also play an important role in Finca los Nacientes frogs' markedly effective inhibition. Although these findings from both mite- and ant-

derived alkaloids suggest that alkaloid quantity contributes more to microbial growth inhibition than alkaloid diversity, the acquisition of such extreme quantities of alkaloid defenses presumably takes years to accrue in individual frogs. Interestingly, inhibition by La Selva frogs indicates that tremendous quantities of alkaloids, such as those present in frogs from Finca los Nacientes and Tortuguero, are not necessary for comparable protection.

In the present study, alkaloid diversity varied considerably, from an average of 26 unique alkaloids in Gandoca frogs to 54 unique alkaloids in Finca los Nacientes frogs, although Tortuguero, La Selva, and Río Palmas frogs contained the average 38 unique alkaloids per frog (**Table 4**). The alkaloid defenses of frogs from La Selva and Gandoca were virtually identical in terms of average quantity, but Gandoca alkaloids were about 25% less diverse than those from La Selva, representing a unique opportunity to compare microbial growth inhibition and alkaloid diversity with quantity held constant. In both OD and CFU assays with *A. hydrophila*, Gandoca frog alkaloids were the least inhibitory of all populations, in accord with their low diversity. In contrast, La Selva frog alkaloid cocktails inhibited *A. hydrophila* virtually as well as those from Finca los Nacientes and Tortuguero, despite having 300% lower quantities of less diverse alkaloids (**Fig. 4**; **Table 4**). This may indicate that microbial inhibition increases at a progressively reduced rate after a certain threshold in the quantity and/or diversity of alkaloids (e.g., average quantity and diversity of La Selva frogs), and also clearly demonstrates that increased alkaloid diversity has the potential to considerably enhance microbial growth inhibition.

In certain cases, however, a high diversity of alkaloids may not be enough to compensate for low quantities of alkaloids. For instance, the alkaloid cocktails of Río

Palmas frogs were marginally more diverse than those from Tortuguero frogs (**Table 4**), yet inhibited growth of *K. pneumoniae* the least effectively of all populations (**Fig. 5**). Such substantially reduced growth inhibition of *K. pneumoniae* by Río Palmas frog alkaloids was most likely a result of having the lowest quantities of alkaloids of all populations, despite their high diversity (**Table 4**). In this case, the average quantity of alkaloids in Río Palmas frogs may have been comparatively far below the hypothetical threshold quantity after which gains in microbial inhibition diminish. Moreover, if such a threshold quantity exists, then particular alkaloid classes are likely required rather than any arbitrary suite of alkaloids. Coincidentally, the already low quantities of alkaloids in Río Palmas frogs were also evenly distributed among the identified alkaloid structural classes, which was not the case in other populations (**Table 1**). For example, the five most abundant alkaloids among Río Palmas frogs were each of a different structural class and comprised just 28% of the population's total ~3,700 µg of alkaloids, whereas the five most abundant alkaloids in the more inhibitory Tortuguero frog alkaloid cocktails comprised just the two most common structural classes (5,8-disubstituted and 5,6,8-trisubstituted indolizidines) and 62% of Tortuguero's total ~14,000 µg of alkaloids (**Table 1**). Accordingly, optimal growth inhibition likely requires certain quantities of specific types of alkaloids, although even comparatively low quantities (e.g., Río Palmas) and diversities (e.g., Gandoca) of alkaloids can inhibit the growth of these pathogens to an extent.

Alkaloid defenses from all five populations of *O. pumilio* effectively inhibited growth of the opportunistic, gram-negative bacteria *A. hydrophila* and *K. pneumoniae*, although OD and CFU assays suggest slightly different interpretations of the results

(**Figs. 4 and 5**). For example, growth inhibition approximated by OD assays indicated that certain populations of *O. pumilio*, such as Río Palmas and Gandoca, did not effectively inhibit the growth of these pathogens compared to control, which may indicate that they are susceptible to infection. However, as evident from CFU assays, treatment with alkaloid cocktails from these same locations significantly reduced the amount of viable bacterial cells persisting after 24.5 hours in *A. hydrophila* and *K. pneumoniae* cultures compared to control, which suggests that alkaloid cocktails from Río Palmas and Gandoca are effective antimicrobial agents. These observations of effective growth inhibition in CFU assays but not OD assays demonstrate the value of CFU assays, and may in fact provide a more ecologically valid assessment of antimicrobial efficacy. The discrepancies in effective growth inhibition between OD and CFU assays for Río Palmas and Gandoca frogs are likely explained by each assay's measured growth variable; OD assays measured the density of both live and dead cells suspended in solution, whereas CFU assays evaluated the quantity of viable (i.e., able to multiply via binary fission) bacterial cells remaining after treatment with *O. pumilio* alkaloid cocktails. Moreover, OD assays and CFU assays were strongly correlated (**Fig. 8**), and the relative patterns of growth inhibition among populations are nearly identical between OD assays and CFU assays, wherein the most (Finca los Nacientes and/or Tortuguero) and least inhibitory (Río Palmas and/or Gandoca) populations were consistent between assays for each pathogen (**Figs. 4 and 5**).

On the basis of the relationships among *O. pumilio* alkaloid quantity, diversity and microbial growth inhibition in the present study, the efficacy of alkaloid-based microbial growth inhibition appears to increase most notably when alkaloid defenses

comprise sufficient (e.g., >400 µg per frog skin) quantities of diverse suites of alkaloids (e.g., >30 individual alkaloids), and when there are high quantities of certain types of alkaloids. Consequently, although the general quantity and diversity of alkaloids in poison frog defenses undoubtedly drive variation among populations, the presence of specific types of alkaloids also likely plays an important role in microbial growth inhibition. Overall, these findings support the hypothesis that alkaloid defenses from distinct populations of *O. pumilio* inhibit pathogens to different degrees, which may influence how those populations respond to emerging infectious pathogens.

Mina *et al*. (2015) reported similar findings, concluding that the only one of their five studied *O. pumilio* populations to effectively inhibit growth of all three of the non-pathogenic microbes (*B. subtilis*, *E. coli*, and *C. albicans*) also had the highest quantity and diversity of alkaloids. On the basis of findings from the present study and Mina *et al*. (2015), poison frogs that sequester greater quantities and types of alkaloids (e.g., longer-lived individuals that have accrued more alkaloids than younger sympatric individuals, or populations that have access to arthropods with more and diverse alkaloid defenses) may benefit from a more effective antimicrobial defense (Jeckel *et al*., 2015b). However, the composition of alkaloid cocktails from *O. pumilio* in the present study suggests that alkaloid diversity accumulates rapidly in individual frogs and levels off between 40 and 60 alkaloids, whereas alkaloid quantity increases indeterminately at a reduced rate (**Fig. 3**). Diversity is presumably limited as such because the assemblages of available dietary arthropods produce a finite number of unique alkaloids. Moreover, since growth inhibition increases with alkaloid diversity (**Figs. 6 and 7**; Mina *et al*., 2015), this diversity limit may also entail diminishing gains in microbial inhibition as the maximum

diversity is approached. Conversely, the quantity of alkaloid defenses in individual *O. pumilio* is most likely limited only by the number and size of granular glands in the skin (Saporito *et al*., 2010b), although a maximum alkaloid volume has not yet been described. As a result, it is feasible that alkaloid quantity could virtually accrue indefinitely. While massive quantities of alkaloids would in all probability only increase the antimicrobial efficacy of a frog's alkaloid defenses, results from the present study seem to indicate that inhibitory activity is increased at progressively reduced rates past a certain quantity. Of course, this hypothetical threshold quantity would likely be entirely dependent on the specific alkaloids present in skin secretions, as well as the identity of the invading pathogen.

Individual alkaloids inhibit the growth of the bacteria *B. subtilis* and *E. coli* to varying degrees or not at all (Macfoy *et al*., 2005), which indicates that alkaloid types differ in their inhibitory activity against specific types of microbes (e.g., gram-negative or gram-positive bacteria, or various fungi, viruses, etc.). For instance, Macfoy *et al*. (2005) report that nearly all individual assayed alkaloids inhibited the growth of *B. subtilis*, whereas only a piperidine alkaloid inhibited *E. coli*. Similarly, certain individual pyrrolidine, piperidine, decahydroquinoline, and pumiliotoxin alkaloids effectively inhibited growth of the fungus *C. albicans* (Macfoy *et al*., 2005). In contrast, Mina *et al*. (2015) reported that *C. albicans* was inhibited only by Isla Solarte (Panama) frog alkaloid cocktails containing primarily decahydroquinolines and pumiliotoxin alkaloids, although alkaloid cocktails from Puerto Viejo (Costa Rica) frogs, consisting of large amounts of decahydroquinolines and histrionicotoxins, also inhibited *C. albicans* somewhat effectively. These findings are consistent with results from the present study in

concluding that certain alkaloid classes such as decahydroquinolines and histrionicotoxins, which are unbranched-chain alkaloids derived from ants (Spande *et al*., 1999; Daly *et al*., 2005; Jones *et al*., 2012), may be particularly inhibitory. However, microbial inhibition by alkaloid defenses appears to be influenced by more than just the presence of specific alkaloid types.

Important complementary and/or synergistic relationships among sequestered alkaloids may enhance protection from pathogens in poison frogs. For example, while the aforementioned individual alkaloids differ in terms of which types of bacteria they can inhibit (Macfoy *et al*., 2005), natural alkaloid cocktails from *O. pumilio* that contain a diversity of alkaloids consistently inhibit the same bacteria (Mina *et al*., 2015). In synthesized amphibian chemical defenses, such as antimicrobial peptides, a combination of various peptides is more effective than the individual peptides at inhibiting pathogenic microbes, including *A. hydrophila* (Rollins-Smith *et al*., 2002b; Rollins-Smith, 2005; Rosenfeld *et al*., 2006; Conlon, 2011a). Additionally, more diverse suites of peptides have been proposed to provide protection from a wider range of pathogens by enhancing other peptides' inhibitory activity (i.e., synergy), or by different peptides or classes targeting different classes of microbes (i.e., complementary) (Nicolas and Mor, 1995; Simmaco *et al*., 1998b; Zasloff, 2002; Tennessen *et al*., 2009). Based on previous alkaloid-based microbial inhibition research and relationships between inhibition and alkaloid composition from the present study, similar synergistic and/or complementary effects may occur in sequestered alkaloid defenses, where comparatively diverse alkaloid cocktails provide enhanced protection from a wider range of pathogens.

Relatively little is known about the antimicrobial function of sequestered alkaloids in the skin of dendrobatids. However, synthesized antimicrobial peptide defenses are common among amphibians, and are considered effective as an innate defense against pathogens and in preventing wound infection (Zasloff, 1987; Nicolas and Mor, 1995; Simmaco *et al*., 1998b). Frog species with greater quantities of diverse suites of antimicrobial peptides appear to be better protected (Zasloff, 2002; Tennessen *et al*., 2009), and the expression of antimicrobial peptides defenses increases following infection (Miele *et al*., 1998; Pask *et al*., 2012). Although antimicrobial peptides in *Rana pipiens* have been shown to degrade fairly quickly (i.e., active for 15 minutes to 2 hours), this is hypothesized to represent a compromise between limiting pathogen infection intensity in critical situations and harm to residential, potentially beneficial microbiota (Woodhams *et al*., 2007b; Conlon, 2011b; Pask *et al*., 2013). The duration that secreted alkaloids remain on the skin is not yet known in dendrobatids, although alkaloids are unlikely to degrade similarly to antimicrobial peptides and would likely represent a comparatively constant first line of defense against microbial pathogens encountered in the environment. While most amphibian antimicrobial peptides form pores in the plasma membrane of gram-negative bacteria to disrupt cellular membranes and subsequently disintegrate the cells (Yang *et al*., 2001; Brogden, 2005; Diamond *et al*., 2009; Chen *et al*., 2012), little is known about the antimicrobial capacity and mechanism of action of poison frog alkaloids.

Lipophilic alkaloids in poison frogs vary in their neuromuscular modes of action by structural class, but in general block or prevent the inactivation of sodium channels and nicotinic acetylcholine receptors (Daly and Spande, 1986; Daly *et al*., 1999;

reviewed in Santos *et al*., 2016). The various classes of alkaloids may have antimicrobial mechanisms of action that correspond to those observed in muscles and neurons, or they may act in a yet unknown way to disrupt normal bacterial processes. Moreover, classes of alkaloids that differ in their mechanism of action most likely vary in their antimicrobial efficacy and the type of microbes they are effective against (e.g., gram-positive or gram-negative bacteria, fungi, or viruses, which differ widely in structure). Lipophilic alkaloids are not amphipathic like antimicrobial peptides and therefore cannot induce pore formation to disrupt microbial membranes. However, much like lipid-binding properties enhances cytotoxicity in antimicrobial peptides (Mahalka and Kinnunen, 2009), the lipophilic properties of sequestered alkaloids may facilitate binding to microbial cell membranes and/or uptake into the cells. Sequestered alkaloids may therefore be able to depolarize bacterial membranes by binding to various ion channels (Saimi *et al*., 1998), which could result in solute influx or efflux that disrupts microbial cells, or they could possibly have intracellular targets that influence their antimicrobial activity. For example, many alkaloids act as antimicrobials through the inhibition of efflux pumps (i.e., active transport pumps) (Markham *et al*., 1999; Maurya *et al*., 2013; reviewed in Cushnie *et al*., 2014), and certain indolizidine alkaloids have been proposed to inhibit nucleic acid synthesis (Rao and Vekatachalam, 2000). Although very little is known about the antimicrobial activity of poison frog alkaloids, these types of microbial interactions may allow frog alkaloids to kill bacteria directly (i.e., bactericidal action) and/or inhibit bacterial reproduction (i.e., bacteriostatic action).

Alkaloid defenses in poison frogs may provide dual predator and pathogen protection. For example, prospective predators that sample and ultimately reject poison

frogs as a prey item (Brodie and Tumbarello, 1978; Fritz *et al*., 1981; Szelistowski, 1985; Gray *et al*., 2010; Stynoski *et al*., 2014; Hantak *et al*., 2016; Hovey *et al*., 2016; Murray *et al*., 2016) stimulate the secretion of defensive alkaloids onto the skin, and may inflict wounds that could be potentially fatal if infected. In this case, poison frog alkaloid secretions could potentially reduce wound infection and inhibit the growth of non-residential microbes including pathogens. However, it should be noted that a resident microbiota community almost certainly persists in the presence of alkaloid defenses (Culp *et al*., 2007; Lauer *et al*., 2007; Walke *et al*., 2015). Additionally, it is feasible that incipient infections in poison frogs may elicit a stress response that stimulates smooth muscle around granular glands, thereby inducing the secretion of additional alkaloids onto the skin to reduce the viable infection load. For instance, *Rana esculenta* and *Bombina orientalis* induce and/or upregulate synthesized antimicrobial peptide defenses after exposure to microbes, including *A. hydrophila* (Miele *et al*., 1998; Simmaco *et al*., 1998a; Mangoni *et al*., 2001). Sequestration of arthropod-derived alkaloids by poison frogs may therefore be analogous in some ways to synthesized peptide defenses (Zasloff, 1987; Pask *et al*., 2013) and play an important role in protection from infectious pathogens, especially when the innate immune system is compromised or the frog is wounded.

Amphibians are inhabited by communities of microbes (e.g., gut-associated microbes and skin-associated microbes), some of which are symbiotic and complement their innate immune system by defending the host from infectious pathogens (Culp *et al*., 2007; Lauer *et al*., 2007, 2008; Woodhams *et al*., 2007c; Myers *et al*., 2012; Park *et al*., 2014; Becker *et al*., 2015). Conversely, certain gram-negative gut- or skin-resident

microbes that are frequently isolated from wild and captive frogs, such as *A. hydrophila* and *K. pneumoniae* (Hunsaker and Potter, 1960; Lee and Franz, 1973; Hird *et al*., 1981, 1983; Bradford, 1991; Barra *et al*., 1998), can cause fatal systemic infections (i.e., "red-leg syndrome"; Rigney *et al*., 1978; Taylor *et al*., 2001) in immunocompromised amphibians (Carr *et al*., 1976; Hubbard, 1981; Pearson, 1998). Of these microbes, *A. hydrophila* in particular has been linked to wild and captive population amphibian declines (Nyman, 1986; Pearson *et al*., 2000; Huys *et al*., 2003; Miller *et al*., 2008; Hill *et al*., 2010). The resistance and resulting virulence of *A. hydrophila* appears to be due to its production of extracellular proteases that break down amphibian antimicrobial peptides, rendering these synthesized chemical defenses largely ineffective (Rollins-Smith *et al*., 2002a; Schadich and Cole, 2009). Metabolites of *Phyllomedusa distincta* skin microbiota also effectively inhibited growth of *E. coli* and other microbes in an optical density-based growth inhibition assay, whereas *A. hydrophila* was most resistant to growth inhibition. Additionally, *K. pneumoniae* was not inhibited by the same metabolites, although in a separate streak assay (Brito de Assis *et al*., 2016). In the present study, alkaloid cocktails from all populations of *O. pumilio* clearly inhibited the resistant pathogen *A. hydrophila* and the relatively resistant, occasionally more pathogenic, *K. pneumoniae*. The conclusion that poison frog alkaloids can reliably inhibit *A. hydrophila* may indicate that the extracellular proteases produced by the pathogen are inactive against sequestered alkaloids, especially given that those proteolytic compounds confer *A. hydrophila* resistance to antimicrobial peptides (Rollins-Smith *et al*., 2002a; Tennessen *et al*., 2009; Brito de Assis *et al*., 2016). This is consistent with the hypothesis that *A. hydrophila* proteases coevolved specifically with widespread amphibian chemical defenses, such as

synthesized peptides or microsymbiont-derived metabolites, as an adaptation to survive on amphibian skin (Schadich and Cole, 2009). Alternatively, the mechanism of action of poison frog alkaloids may simply be more effective against *A. hydrophila* and *K. pneumoniae* than that of the antimicrobial peptides and metabolites studied so far. However, the same antimicrobial peptides that do not inhibit *A. hydrophila* can effectively inhibit the pathogen most closely associated with global amphibian population declines and biodiversity loss, *Batrachochytrium dendrobatidis* (Berger *et al*., 1998; Rollins-Smith *et al*., 2002b). The inhibitory activity of sequestered alkaloid defenses against *Bd* is not yet known. Thus, although findings from the present study demonstrate that poison frog alkaloids can function as an effective defense against virulent pathogens, the inhibitory activity of alkaloid defenses against other highly-relevant pathogens remains to be determined.

The chytrid fungus *Bd* is the primary pathogen implicated in disease-related amphibian declines (Blaustein *et al*., 2012). Nonetheless, various other pathogens including ranaviruses, water molds, and mycobacteria frequently co-occur with chytrid and one another (Miller *et al*., 2008; Hill *et al*., 2010; Prada-Salcedo *et al*., 2011; Hoverman *et al*., 2012; Souza *et al*., 2012; Whitfield *et al*., 2013; Warne *et al*., 2016), and likely contribute to disease dynamics and amphibian population declines. Numerous poison frog species are endemic to habitats in which these pathogens are prevalent and amphibian population declines have been observed (Hunter *et al*., 2010a; Catenazzi *et al*., 2011; Whitfield *et al*., 2012; Agostini *et al*., 2015; Rodríguez-Brenes *et al*., 2016). Moreover, although the Malagasy lineage of *Mantella* poison frogs is endemic to localized habitats that have not yet experienced pathogen-related amphibian declines, low

infection loads of *Bd* and ranavirus infection have recently been detected in sympatric native amphibians, and these habitats are climatically suitable for the establishment and potential outbreak of the aforementioned pathogens (Kolby, 2014; Bletz *et al*., 2015; Kolby and Skerratt, 2015; Kolby *et al*., 2015). The presence of *Bd* and other pathogens is also unlikely to be eliminated from the environment, so effective treatment strategies for a range of pathogens are imperative in order to facilitate the persistence of natural frog populations *in situ*, and mitigate amphibian biodiversity decline. Treatment with antimicrobial peptides appears to be an effective treatment for clearing infection in diseased amphibians, particularly for those lacking antimicrobial peptides (Woodhams *et al*., 2012). Conversely, although these treatments are beneficial for treating infected amphibians, they do not provide an effective long-term survival solution. Probiotic bioaugmentation, wherein amphibians are supplemented with cutaneous bacteria that produce antimicrobial metabolites and promote survival in infected frogs (Lam *et al*., 2010; Becker *et al*., 2011; Flechas *et al*., 2012; Woodhams *et al*., 2014), has been proposed as a feasible *in situ* and *ex situ* conservation approach to mitigate disease prevalence and mortality in at-risk amphibian species and communities (Muletz *et al*., 2012; Bletz *et al*., 2013; Woodhams *et al*., 2016). However, poison frogs, which effectively lack synthesized peptide defenses (Daly *et al*., 1984, 1987; Erspamer *et al*., 1986; Prates *et al*., 2012) and have not been examined for bacterial communities, may resist or be less receptive to probiotic bioaugmentation as a consequence of the toxic sequestered alkaloids in their skin secretions.

A prospective alternative for endangered or at-risk poison frogs may be dietary supplementation with alkaloids, which would facilitate the accumulation of sequestered

defenses. Based on results from this study, alkaloid supplementation could potentially inhibit the growth of pathogens in captive poison frogs and upon reintroduction to their natural habitat. Especially considering that the amphibian immune system is significantly impaired during metamorphosis (Rollins-Smith, 1998), such an approach would be particularly important for poison frogs raised in captivity (e.g., certain *ex situ* conservation programs, or zoo collections), which would not have had access to alkaloid-containing dietary arthropods that occur in their natural habitat. In some amphibians, synthesized peptides and bacterial symbionts provide protection from disease in embryonic, larval, and juvenile life stages (Banning *et al*., 2008; Walke *et al*., 2011; Pask *et al*., 2013; Holden *et al*., 2015). However, comparable defenses have not been identified in poison frogs, which underscores the importance of their sequestered alkaloids, particularly in earlier life stages. For instance, maternal provisioning of alkaloids via nutritive eggs is the sole source of alkaloid defenses for dendrobatid poison frog tadpoles of the genus *Oophaga*, wherein these provisioned alkaloids provide critical early protection from predators (Stynoski *et al*., 2014). On the basis of the present study, provisioned alkaloids may also reduce susceptibility to microbial pathogens through the critical transition to metamorphosis. Although maternal provisioning of alkaloids appears to be unique to members of the genus *Oophaga* poison frogs, it is conceivable that analogous alkaloid supplementation of other poison frogs in the early stages of life would enhance resistance to infectious pathogens, which is critical for endangered poison frogs.

A variety of poison frogs are represented in conservation programs (McFadden *et al*., 2013; Edmonds *et al*., 2015), but little is known about how sequestered alkaloids protect them from infectious pathogens, or how alkaloids may be utilized to their

advantage. For example, certain Australian *Pseudophryne* frogs were extirpated from the wild by *Bd* and are fortunately represented in *ex situ* conservation programs (McFadden *et al*., 2013; Brannelly *et al*., 2015a, 2015b). Pseudophrynaminol, a synthesized alkaloid unique to this genus (Daly *et al*., 2005), does not inhibit the growth of *B. subtilis*, *E. coli*, or *C. albicans* (Macfoy *et al*., 2005) or elicit toxicosis in the fire ant *S. invicta* (Weldon *et al*., 2013), suggesting that its synthesized alkaloid defenses may not be particularly useful in inhibiting growth of pathogenic microbes and subsequently in facilitating successful reintroductions. However, since *Pseudophryne* spp. are also capable of sequestering pumiliotoxin alkaloids (Daly and Garraffo, 1990), future studies could assess whether sequestered alkaloids, or synthesized pseudophrynamines, are effective at inhibiting pathogens such as *Bd*, which may inform beneficial captive management strategies. Aside from the critically endangered *Pseudophryne* frogs, other species and populations of poison frogs are surely susceptible to pathogens. Future research could therefore include assessments of susceptibility from a wide range of lineages and populations of poison frogs. Electrical stimulation of granular glands to secrete alkaloids may be used as a minimally invasive field method of evaluating the composition and antimicrobial capacity of poison frog alkaloid defenses (Grant and Land, 2002; Daly *et al*., 2008a; Clark, 2010; Hantak *et al*., 2013). Overall, assessments of the antimicrobial efficacy of natural poison frog alkaloid defenses would undoubtedly be useful in determining prioritization strategies for conservation management of poison frogs now and in the future, when interactions between threats like continued habitat loss, climate change, and spreading infectious diseases may accelerate amphibian declines.

Research on synthesized amphibian chemical defenses in recent decades has developed our understanding of host-pathogen interactions extensively, but similar research on comparatively uncommon sequestered defenses such as alkaloids has just begun. Continued research is needed to examine relationships between sequestered poison frog alkaloid defenses and pathogens implicated in amphibian declines in order to determine which aspects of alkaloid defenses contribute most to growth inhibition, as well as to assess the practicality and efficacy of alkaloid provisioning for poison frogs of various life stages. Relevant pathogens to prioritize in such studies include *Bd*, ranaviruses, and water molds (*Saprolegnia* spp.), which are frequently observed in disease-induced mortality of amphibians in the field (Pessier, 2002; Prada-Salcedo *et al*., 2011) and have been involved in peptide research (Rollins-Smith, 2009). Thus far, the majority of microbial inhibition research with amphibian chemical defenses has utilized OD assays to assess growth inhibition. Accordingly, comparisons between OD and CFU assay results in the present study may also inform experimental design decisions in future research. For instance, since certain populations of *O. pumilio* inhibited growth compared to control treatments in CFU assays but not in the preceding OD assays (**Figs. 4 and 5**), assessments of the antimicrobial efficacy of amphibian chemical defenses may require CFU assays following OD assays to accurately determine whether the compounds inhibited growth compared to control treatments. Conversely, since the relative patterns of growth inhibition among populations are nearly identical between OD assays and CFU assays for these pathogens (**Figs. 4 and 5**; **Fig. 8**), OD assays alone may be sufficient to estimate and compare the antimicrobial capacity of chemical defenses of different types or from various amphibian populations or taxa.

In the present study, results from these microbial growth inhibition assays serve as a step towards understanding whether poison frog alkaloid defenses may have evolved as a means of protection against pathogenic microbes. Much like variation in alkaloid defenses is hypothesized to play a role in defense against predators (Saporito *et al*., 2006, 2007a; Murray *et al*., 2016), these findings suggest that the natural variation in poison frog alkaloid defenses influences protection from pathogenic microbes. As a result of comparable variation in dietary arthropods (Clark *et al*., 2006; Bonansea and Vaira, 2007; Daly *et al*., 2008a; Quiroga *et al*., 2011; Andriamaharavo *et al*., 2015), similar trends in microbial inhibition would likely be observed in other poison frog lineages, some of which are endangered due to threats such as continued habitat loss and spreading disease and would likely benefit from conservation management in captive breeding programs. Accordingly, findings from the present study may also be useful in improving conservation programs and strategies for poison frogs.

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Table 1. The five most abundant alkaloids (in terms of relative quantity) identified in the skin of *Oophaga pumilio* from each population.

Population	Arthropod source of alkaloids				Percent of population's total alkaloids
	Mites		Ants		
	Structural class	Alkaloid	Structural class	Alkaloid	
Tortuguero	5,6,8-I	**251T**, **223A**			62%
	5,8-I	**247E**, **205A**, **207A**			
La Selva	5,8-I	**207A**, **195I**	Lehm	**275A**	48%
	Unclass	**311A**, **209G**			
Finca los Nacientes	5,8-I	**195I**, **207A**	DHQ	**269AB**, **269A**	46%
			HTX	**285C**	
Río Palmas	Izi	**209L**	3,5-P	**251K**	28%
	5,6,8-I	**223A**	Pyr	**197B**	
	Tri	**253S**			
Gandoca	5,8-I	**251N**	Lehm	**275A**	57%
	5,6,8-I	**249C**, **259C**	DHQ	**195A**	

Abbreviations for alkaloid classes are as follows: 3,5-P (3,5-disubstituted pyrrolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); 5,8-I (5,8-disubstituted indolizidine); DHQ (decahydroquinoline); HTX (histrionicotoxin); Izi (izidine); Lehm (lehmizidine); Pyr (pyrrolidine); Tri (tricyclic); Unclass (unclassified).

Table 2. Alkaloids detected in *Oophaga pumilio* arranged by structural class.

	1,4-Q	3,5-I	3,5-P	4,6-Q	5,6,8-I	5,8-I	aPTX	Dehydro-5,8-I	Deoxy-hPTX	DHQ	HTX	Izidine	Lehm	Pip	PTX	Pyr	Spiro	Tri	Unclass	
	231A **233A** **257D**(2) **259E** **279E**	**195B**(2) **223AB**(2) **223R** **275C**	**195F** **223B**(3) **223H**(3) **249I**(2) **251K**(3) **251O** **265J**(3)	**237I** **251Y** **253X** **265DD**	**195G** **221P** **221Q** **223A**(5) **223C** **231B**(3) **235E** **237C**(2) **237L**(3) **249C**(4) **251M**(2) **251S** **251T**(2) **253H**(4) **259C**(2) **263A**(4) **265L**(2) **267J** **267CC** **275E**(6) **277E**(2) **279F** **293C**	**195I**(2) **203A** **205A**(3) **207A**(4) **207Q** **209I** **209S** **217B**(3) **219F**(2) **221I** **223D**(2) **223J**(2) **223AA** **231C**(3) **231G** **233D** **235B**(2) **237D**(2) **239G** **241F** **243B** **245I**(3) **247E**(2) **249O**(2) **251N**(2) **259B** **261D**(4) **263F**(2) **265P** **271A** **273B**(2) **273C** **275F**	**241H** **253A** **267A** **293K**(2) **309D**(2) **323B** **341A**	**201A** **203E** **205L**(2) **221D** **221J** **245F** **265F**(2)	**193F** **207O**	**195A** **211A** **223F** **237U** **251A**(2) **267L**(2) **269AB**(6) **269A**(2) **269B**(4) **271D**(3) **275B**(3)	**283A**(2) **285A**(5) **285C**(2) **287A**(2) **287B**(3) **291A**	**193J** **195K** **209L**(3) **211B**(4) **211F** **221N**(2) **223I** **225G**(2) **239A** **239CC** **247J**(2) **267BB** **291B**	**275A**(5) **277A**(3)	**211I**(2) **211J** **225I**(2) **239I**(2) **239L**(2) **241D**(4) **241G**(2) **253J**(2) **253U** **267K**(5) **267X**(2) **269C**	**225F** **237A**(2) **265D** **277B** **307A**(4) **321A** **323A**(2) **325B**	**183B** **197B**(5) **211T** **225P** **235F** **253F** **253I** **267EE** **277D**(2) **277J** **279M**	**236** **252A**	**207GH**(4) **207R** **219O** **221G** **221M** **221W** **245J** **247N** **249Z** **253S**(5) **261C**(2) **261F** **261H** **275L**	**167H** **195N** **197K** **207N** **209G**(2) **211D** **223L** **235I** **235M** **235S** **245R** **247L** **247P** **247Q** **249N**(2) **251HH** **261K** **261L** **265K** **267I** **267O**(2) **267Q**(6) **267DD** **269E**(3) **269K** **271E** **273F** **275M** **281U** **283I** **291K** **311A** **323I**(2)	
Total	5	4	7	4	23	33	7	7	2	11	6	13	2	12	8	11	1	14	33	203

Alkaloids present in quantities greater than 0.5 µg in at least one frog are listed, except in the case of new alkaloids (see **Table 3**). Alkaloids that are underlined represent tentatively new alkaloids that have not been previously described. Abbreviations for alkaloid classes are as follows: 1,4-Q (1,4-disubstituted quinolizidine); 3,5-I (3,5-disubstituted indolizidine); 3,5-P (3,5-disubstituted pyrrolizidine); 4,6-Q (4,6-disubstituted quinolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); 5,8-I (5,8-disubstituted indolizidine); aPTX (allopumiliotoxin); Dehydro-5,8-I (dehydro-5,8-disubstituted indolizidine); Deoxy-hPTX (deoxy-homopumiliotoxin); DHQ (decahydroquinoline); HTX (histrionicotoxin); Lehm (lehmizidine); Pip (piperidine); PTX (pumiliotoxin); Pyr (pyrrolidine); Spiro (spiropyrrolizidine); Tri (tricyclic); Unclass (unclassified).

Table 3. Retention times (R_t) for newly identified alkaloids from *Oophaga pumilio* (see **Appendix 31** for mass spectral data). The R_t for each alkaloid is accompanied by an approximate "corrected R_t" that corresponds to the retention times in the alkaloid library of Daly *et al*. (2005). Following the methods of Garraffo *et al*. (2012), based on comparisons of R_ts for previously identified alkaloids in the present study with R_ts from Daly *et al*. (2005), alkaloids in the present study eluted approximately 0.34 seconds faster than times listed in Daly *et al*. (2005).

Str. Class	Alkaloid	R_t	Corrected R_t
Unclass	**167H**	5.67	5.33
Unclass	**195N**	7.53	7.19
Unclass	**197K**	7.37	7.03
Dehydro-5,8-I	**203E**	8.98	8.64
Pyr	**225P**	9.70	9.36
Unclass	**245R**	12.04	11.70
Unclass	**247P**	10.77	10.43
Unclass	**247Q**	11.69	11.35
Unclass	**251HH**	11.61	11.27
4,6-Q	**253X**	9.70	9.36
Unclass	**261K**	12.94	12.60
Unclass	**261L**	12.96	12.62
4,6-Q	**265DD**	13.30	12.96
Izi	**267BB**	12.65	12.31
5,6,8-I	**267CC**	12.80	12.46
Unclass	**267DD**	13.49	13.15
Pyr	**267EE**	14.40	14.06
Unclass	**269K**	11.38	11.04
Unclass	**273F**	14.88	14.54
Tri	**275L**	13.92	13.58
Unclass	**275M**	14.01	13.67
Pyr	**277J**	14.28	13.94
Pyr	**279M**	14.33	13.99
Unclass	**281U**	13.88	13.54
Unclass	**283I**	15.56	15.22
Unclass	**291K**	15.75	15.41
Unclass	**311A**	15.22	14.88

Abbreviations for alkaloid classes are as follows: 4,6-Q (4,6-disubstituted quinolizidine); 5,6,8-I (5,6,8-trisubstituted indolizidine); Dehydro-5,8-I (dehydro-5,8-disubstituted indolizidine); Izi (izidine); Pyr (pyrrolidine); Tri (tricyclic); Unclass (unclassified).

Table 4. Diversity (alkaloids per frog skin) and quantity (µg per frog skin) of alkaloid defenses from each population of *Oophaga pumilio*.

	Average diversity	**Average ♀ diversity**	**Average ♂ diversity**	**Total diversity**	**Total ♀ diversity**	**Total ♂ diversity**
Tortuguero	37	39	36	521	272	249
La Selva	33	36	30	458	250	208
Finca los Nacientes	54	55	53	758	441	317
Río Palmas	39	42	36	548	334	214
Gandoca	26	21	31	364	147	217
All populations	**38**	**39**	**37**	**2649**	**1444**	**1205**

	Average quantity	**Average ♀ quantity**	**Average ♂ quantity**	**Total quantity**	**Total ♀ quantity**	**Total ♂ quantity**
Tortuguero	986	1224	748	13807	8567	5239
La Selva	344	462	225	4811	3233	1578
Finca los Nacientes	1181	1219	1130	16528	9750	6778
Río Palmas	264	295	223	3697	2357	1340
Gandoca	344	280	524	4812	1148	3665
All populations	**624**	**677**	**564**	**43656**	**25056**	**18600**

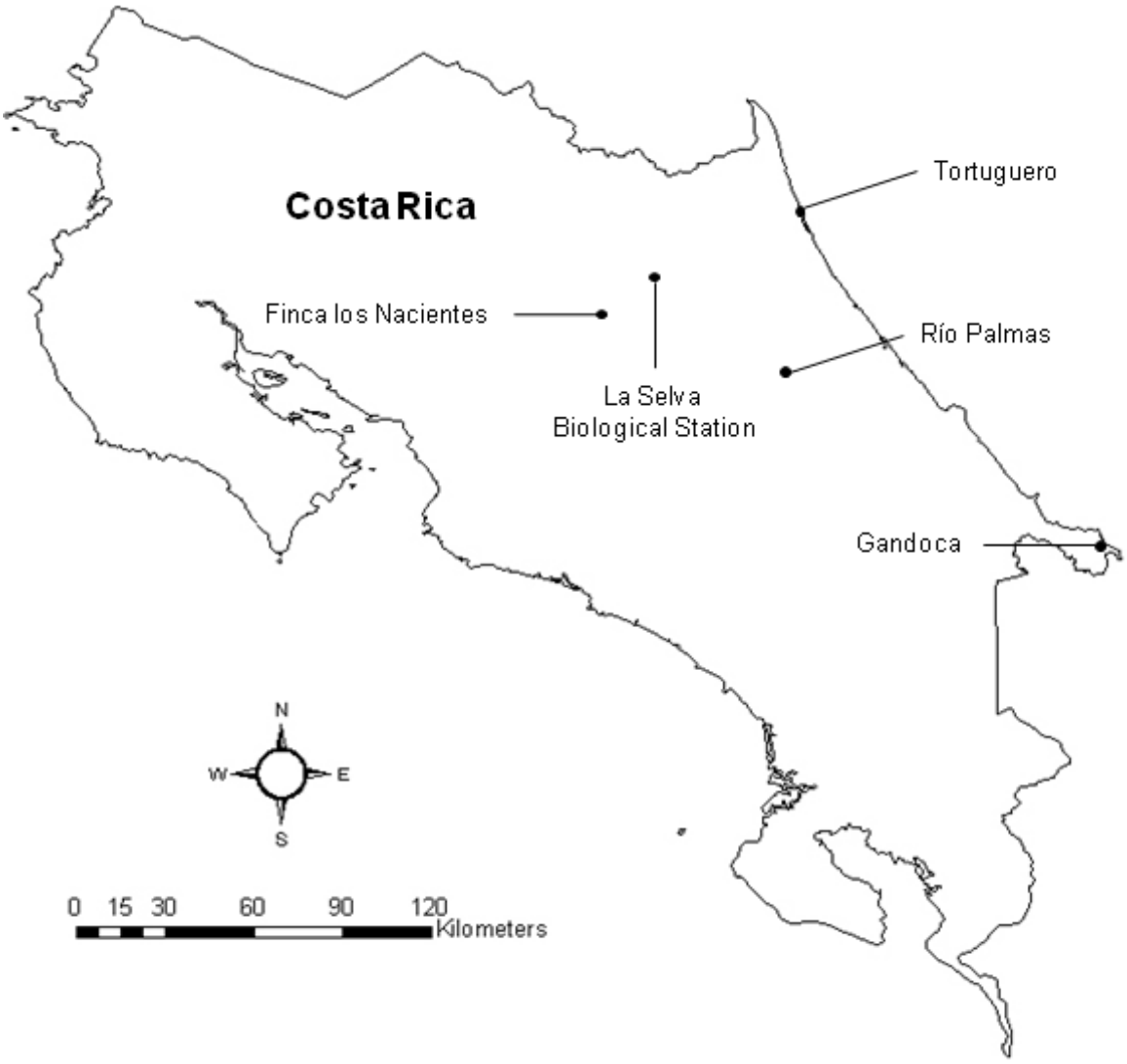


Figure 1. Map of five *Oophaga pumilio* collection sites in Costa Rica.

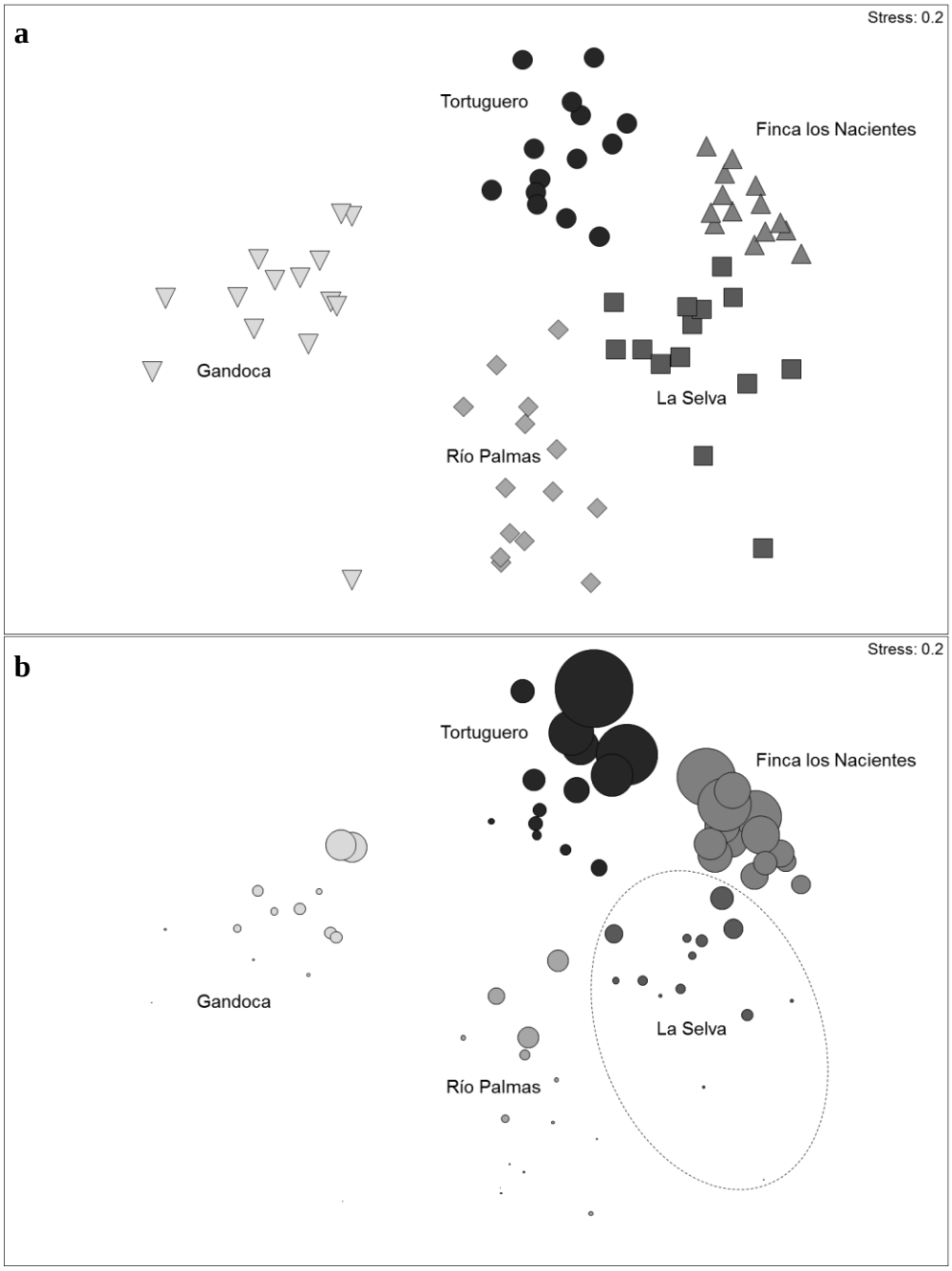


Figure 2. MDS plots [(**a**) original MDS plot; (**b**) scaled to quantity)] of alkaloid composition for five populations of *Oophaga pumilio*. Each point represents an individual frog (70 frogs total, 14 per population). The distance between symbols represents differences in alkaloid composition (number, type, and quantity of alkaloids per frog skin; in (**b**) the diameter of each point is scaled to quantity of alkaloids per frog, and La Selva frogs are encircled by a dotted line to distinguish them from nearby points). Points that are closer together represent frogs that have more similar alkaloid profiles, whereas points that are further apart represent frogs with less similar alkaloid profiles.

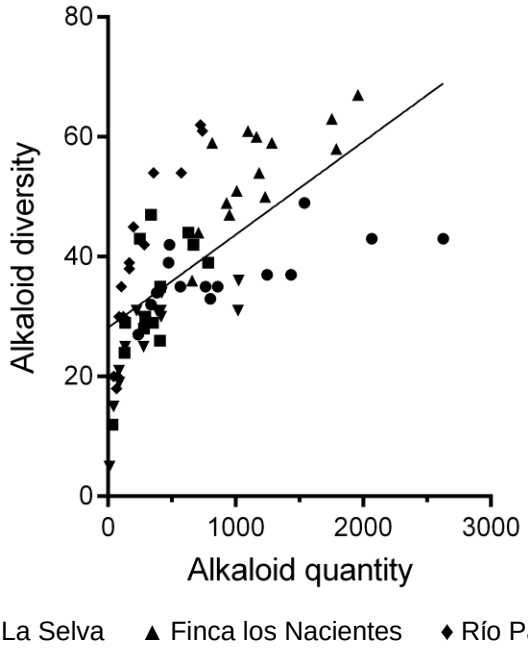


Figure 3. Relationship between alkaloid quantity (µg per frog skin) and diversity (alkaloids per frog skin) in *Oophaga pumilio* from five populations throughout Costa Rica.

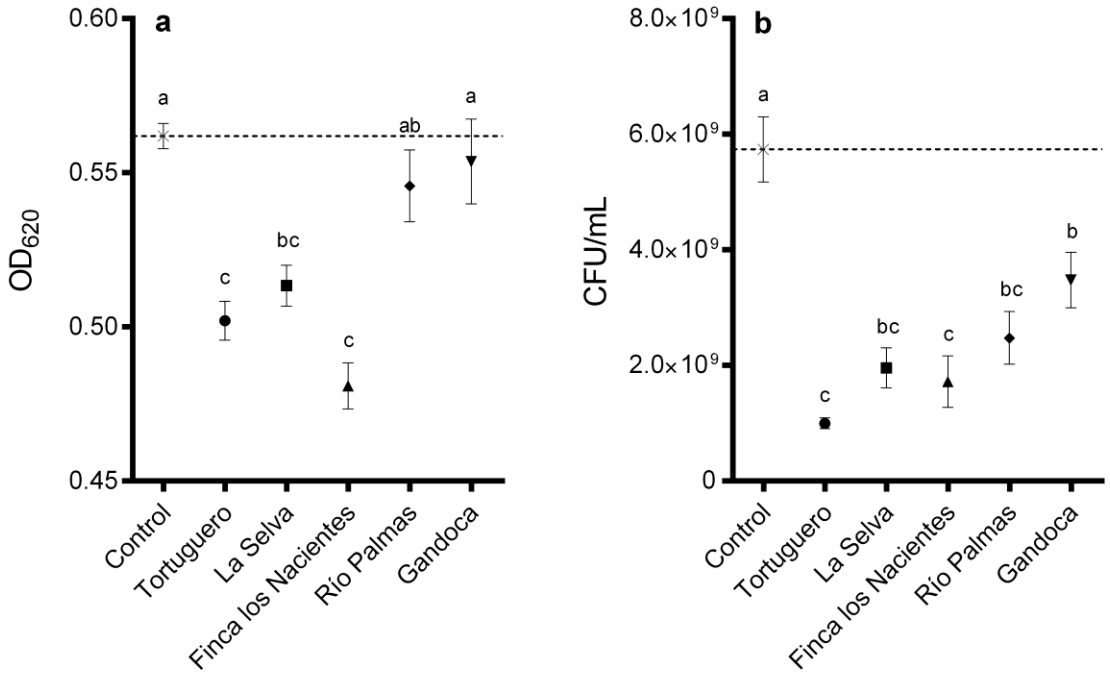


Figure 4. Mean final optical densities (OD_{620}) (**a**) and viable cell densities (CFU/mL) (**b**) of *Aeromonas hydrophila* after treatment with alkaloid cocktails extracted from *Oophaga pumilio*. The dotted line represents the mean OD_{620} and CFU/mL for the methanol control, error bars represent ± 1 standard error of the mean, and treatment means that are significantly different from each other are indicated by different letters (Tukey's HSD, $p < 0.05$).

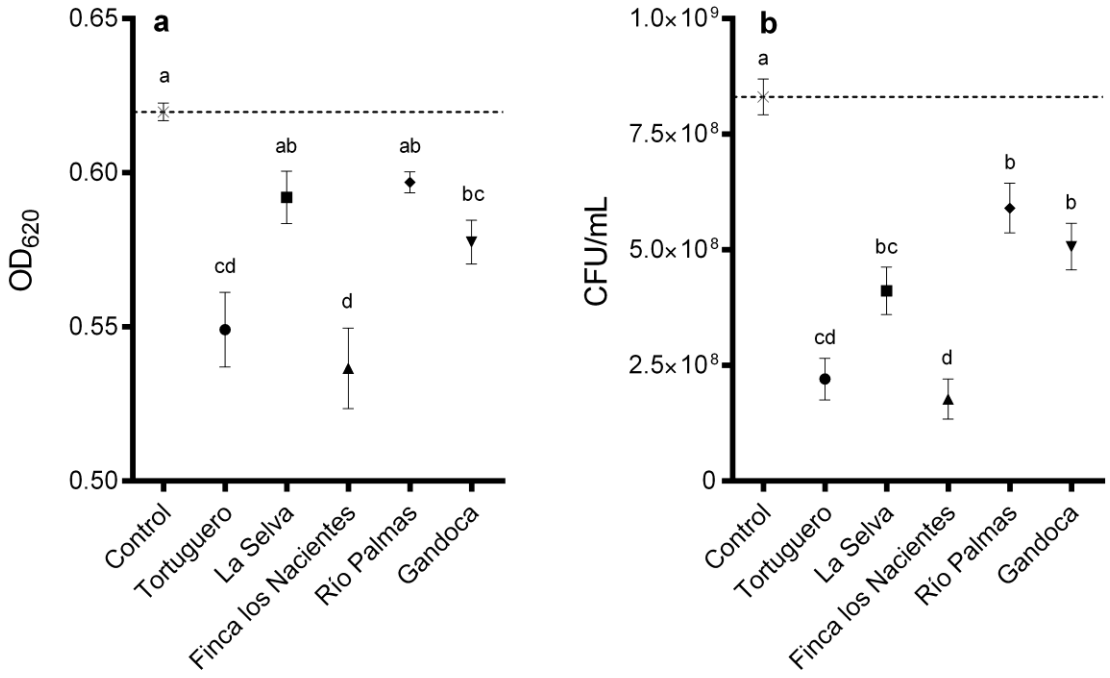


Figure 5. Mean final optical densities (OD_{620}) (**a**) and viable cell densities (CFU/mL) (**b**) of *Klebsiella pneumoniae* after treatment with alkaloid cocktails extracted from *Oophaga pumilio*. The dotted line represents the mean OD_{620} and CFU/mL for the methanol control, error bars represent ± 1 standard error of the mean, and treatment means that are significantly different from each other are indicated by different letters (Tukey's HSD, $p < 0.05$).

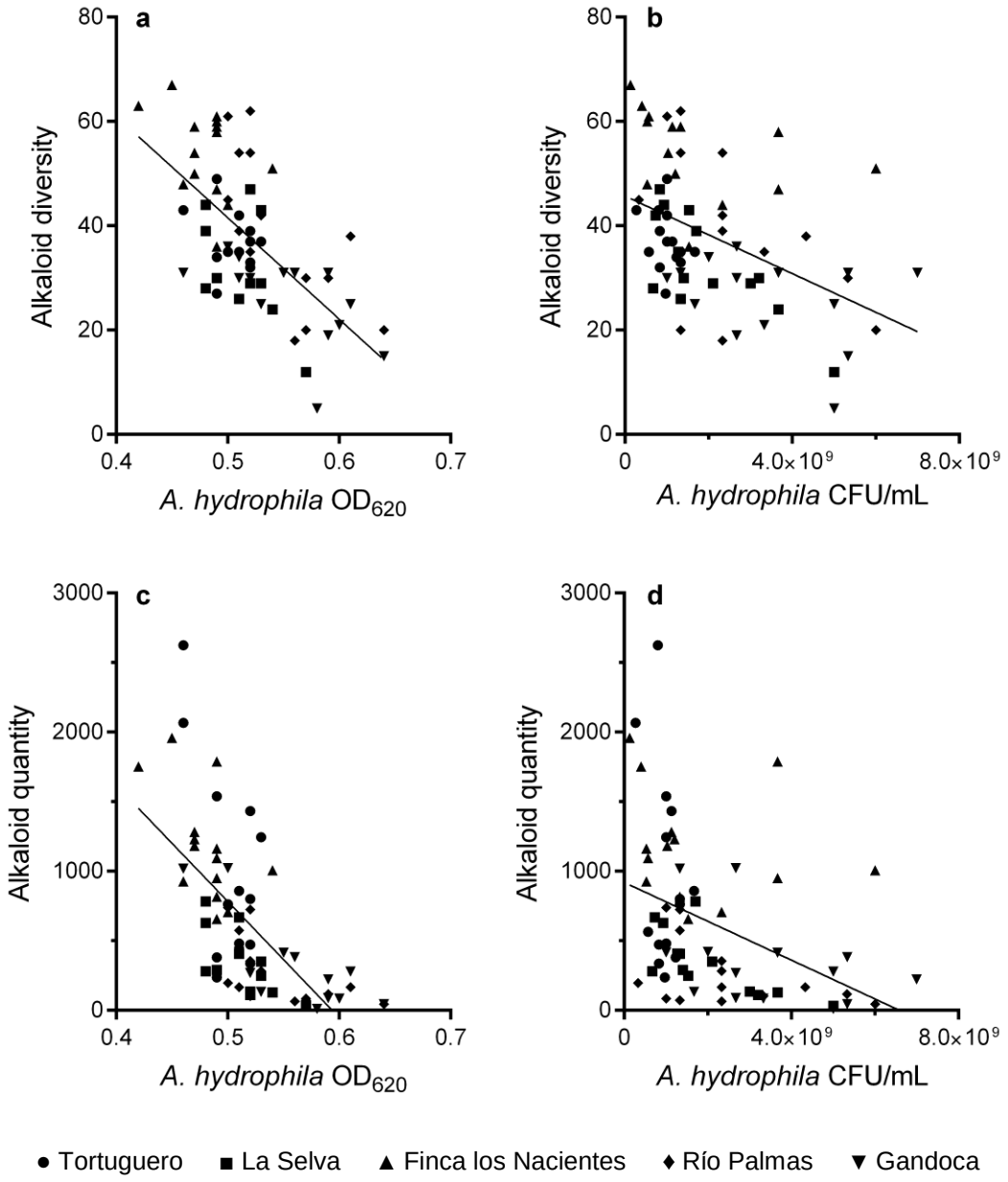


Figure 6. Relationship between alkaloid profiles of individual *Oophaga pumilio* and corresponding growth inhibition of *Aeromonas hydrophila* [(**a**) alkaloid diversity (alkaloids per frog skin) and optical density assay; (**b**) alkaloid diversity (alkaloids per frog skin) and colony-forming unit assay; (**c**) alkaloid quantity (µg per frog skin) and optical density assay; (**d**) alkaloid quantity (µg per frog skin) and colony-forming unit assay].

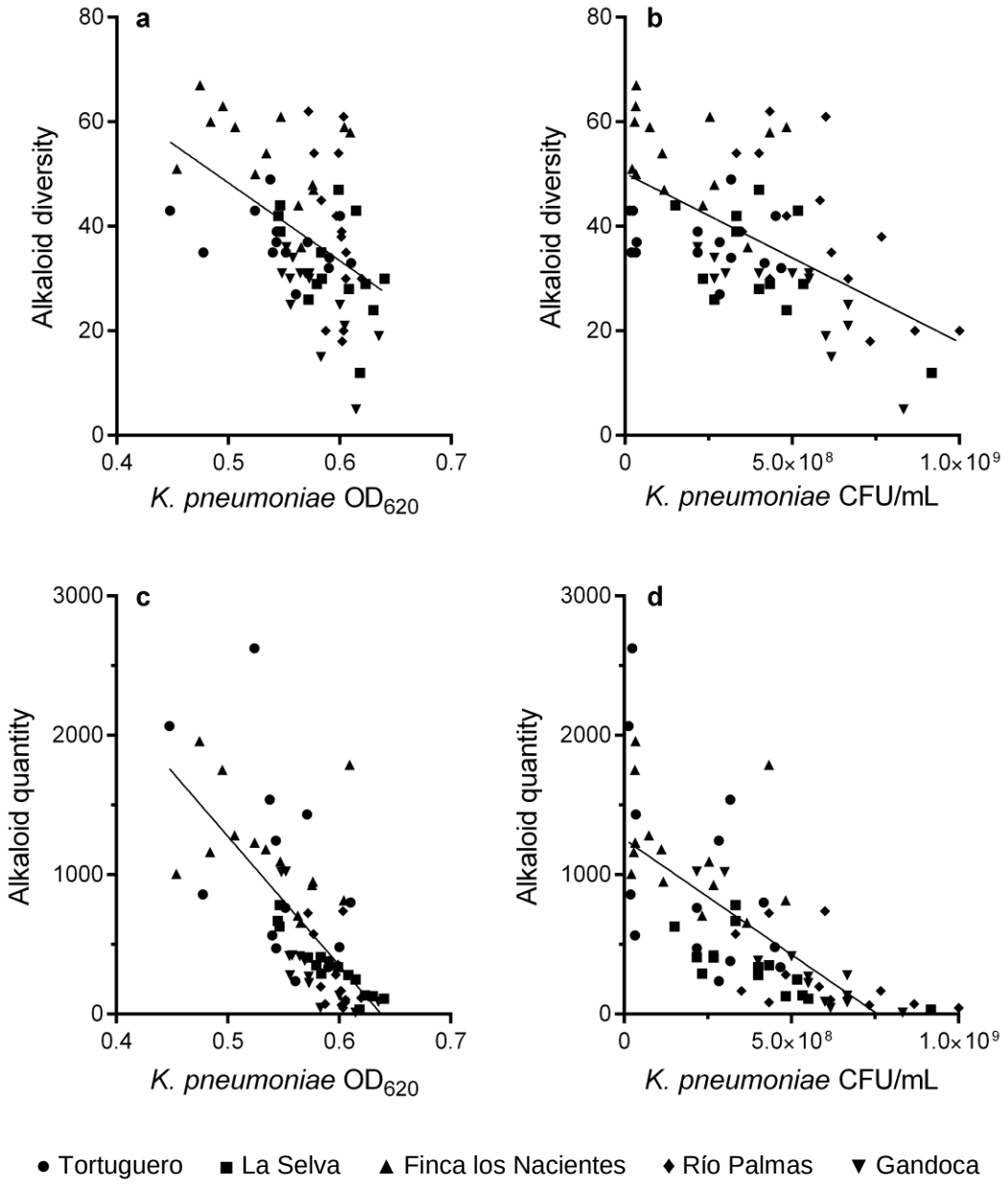


Figure 7. Relationship between alkaloid profiles of individual *Oophaga pumilio* and corresponding growth inhibition of *Klebsiella pneumoniae* [(**a**) alkaloid diversity (alkaloids per frog skin) and optical density assay; (**b**) alkaloid diversity (alkaloids per frog skin) and colony-forming unit assay; (**c**) alkaloid quantity (µg per frog skin) and optical density assay; (**d**) alkaloid quantity (µg per frog skin) and colony-forming unit assay].

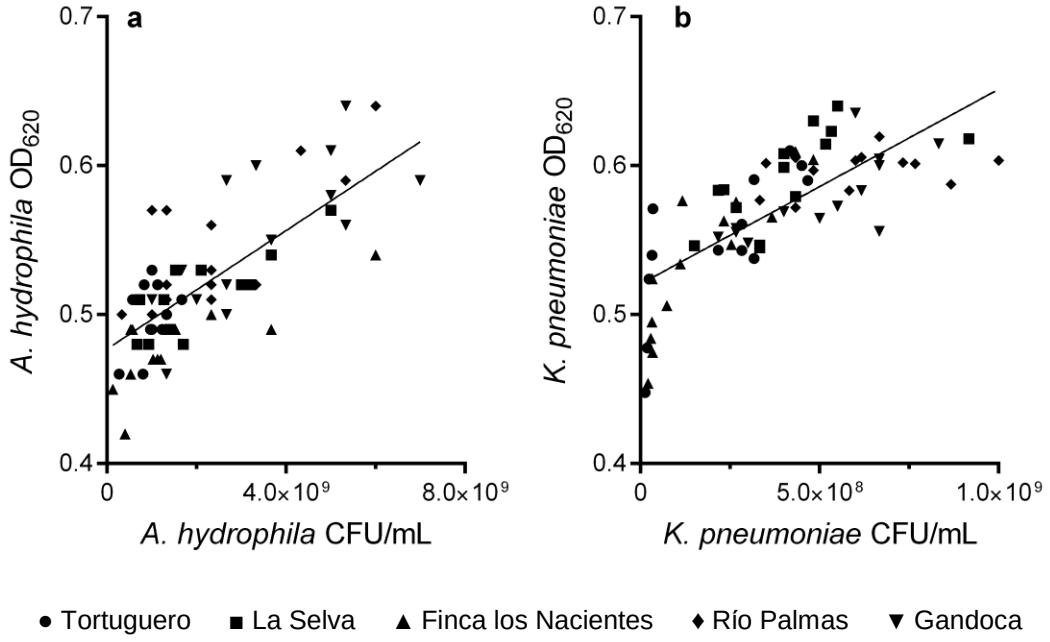


Figure 8. Relationship between optical density and colony-forming unit assays for (**a**) *Aeromonas hydrophila* and (**b**) *Klebsiella pneumoniae* treated with alkaloids extracted from *Oophaga pumilio*.

APPENDIX

Cryopreservation of Microbes

Upon receipt of *Aeromonas hydrophila* (ATCC® 35654™) and *Klebsiella pneumoniae* subsp. *pneumoniae* (ATCC® 13883™), 30 mL TSB stock cultures were grown in 50 mL conical tubes and incubated for 24 hours at 30°C (*A. hydrophila*) or 37°C (*K. pneumoniae*) in a shaking incubator set to 100 rpm. Twelve 1 mL frozen stock cultures were created from the initial 30 mL cultures by adding 900 µL of the culture to 100 µL sterile 80% glycerol in each of twelve labeled cryovials. Frozen stock cultures were stored at -80°C until use in assays.

Pilot Experiments

A variety of preliminary pilot experiments were conducted to determine optimal dilutions and corresponding growth curves of microbial cultures for use in microbial growth inhibition assays, and to examine the effect that various volumes of the vehicle control (methanol) had on the growth of each microbe.

Effect of Methanol on Growth of Various Dilutions of Bacterial Cultures

Aeromonas hydrophila OD Pilot Assays

A 30 mL culture of *A. hydrophila* was grown from an isolated colony on a streak plate and incubated at 30°C with agitation at 100 rpm for 18 hours. Three 10 mL dilutions (1:5, 1:10, and 1:50) were created from this 30 mL culture. For the 1:5 dilution, 2 mL of *A. hydrophila* culture was added to 8 mL TSB. For the 1:10 dilution, 1 mL *A. hydrophila* culture was added to 9 mL broth. For the 1:50 dilution, 0.2 mL of *A. hydrophila* culture

was added to 9.8 mL broth. All dilutions were vortexed for five seconds and then poured into individual plastic troughs immediately prior to transferring aliquots to 96-well microplates. Equal 200 µL volumes (subcultures) of each dilution were immediately plated in a 96-well microplate such that each dilution occupied three columns of eight replicate wells each (total nine columns used per pilot assay). The eight replicate wells in the first of three columns of each of the 1:5, 1:10, and 1:50 dilutions acted as the negative control and were not inoculated, which created a typical growth curve for *A. hydrophila* alone at each dilution. The eight wells in the second columns of each dilution were inoculated with 5 µL of methanol as a proxy for estimating the effect of 4.8 µL of methanol on growth of *A. hydrophila*. The eight wells in the third columns of each dilution were inoculated with 10 µL of methanol as a proxy for estimating the effect of 9.6 µL of methanol on growth of *A. hydrophila*. After preparation, the microplate was placed in the microplate reader and OD_{620} was measured over the course of a 24.5-hour program that recorded OD_{620} at 15-minute intervals and shook the plate at medium speed for five seconds prior to each measurement. All eight OD_{620} measurements for replicate wells for each of the nine treatments were treated as subsamples and were averaged to visualize growth curves.

This pilot assay was conducted twice to verify the repeatability of the results. In both assays, identical trends in *A. hydrophila* growth were observed (**Appendix 1**). The 1:5 and 1:10 dilutions were clearly in mid-log growth phase at the start of the assay and had nearly overlapping growth curves when inoculated with no methanol and 5 µL methanol, although the 1:5 dilution had a slightly higher OD_{620} throughout the 24.5-hour growth period. The 1:50 dilution entered log growth phase after a slight (< 1 hour) delay

compared to the 1:5 and 1:10 dilutions, and had a reduced OD_{620} after 24.5 hours in comparison to the 1:5 and 1:50 dilutions. When inoculated with 10 µL methanol, all dilutions went through a 10 – 12 hour lag phase before entering log growth phase, and grew to a maximum OD_{620} nearly half that of all other growth curves; this suggested that 10 µL methanol was not a feasible vehicle control volume to use in OD assays. Therefore, a pilot experiment comparing 5 µL methanol and 7.5 µL methanol was conducted.

The above procedure was carried out identically for this third pilot assay, but 7.5 µL methanol was used in place of 10 µL methanol. Growth curves for the 1:5, 1:10, and 1:50 dilutions of *A. hydrophila* were identical to the previous pilot assays in treatments when not inoculated with methanol and when inoculated with 5 µL, but the addition of 7.5 µL methanol still delayed log growth by 3 – 5 hours and reduced the final OD_{620} by nearly half (**Appendix 2**). Therefore, 7.5 µL was determined to be an unsuitable vehicle control volume to use in OD assays, and a pilot assay examining the effect of 2.4 µL methanol and 4.8 methanol on growth at each of the three previous dilutions was conducted.

Using the above procedures, growth curves of *A. hydrophila* at 1:5, 1:10, and 1:50 dilutions were identical to those in previous pilots in the absence of methanol and when inoculated with 4.8 µL methanol (**Appendix 3**). Moreover, growth curves of *A. hydrophila* inoculated with 2.4 µL methanol were very similar to growth curves of *A. hydrophila* that had not been inoculated with methanol, which indicated that this volume is ideal for a vehicle control in growth inhibition assays. Based on these growth curves, the 1:10 dilution was determined to be most suitable for growth inhibition assays since it

started at an intermediate OD_{620} compared to 1:5 and 1:50 dilutions and appeared to start at the very beginning of log growth phase in each assay, thereby providing the most appropriate growth curve with which to assess growth inhibition due to alkaloids. Since growth curves of *A. hydrophila* inoculated with 4.8 µL methanol were somewhat inhibited compared to *A. hydrophila* not inoculated with methanol, a pilot assay was conducted to determine whether or not adding methanol in the mid-log phase (after a 1.5 hour delay) would change rates of growth at the mid-log phase in the 1:10 dilution of *A. hydrophila*.

In this pilot assay, *A. hydrophila* was cultured and diluted following the same procedure as previous pilots, but the 1:10 dilution of *A. hydrophila* was examined under five conditions in triplicate: no methanol, 2.4 µL methanol added prior to measurements, 4.8 µL methanol added prior to measurements, 2.4 µL methanol added 1.5 hours after starting the assay, and 4.8 µL methanol added 1.5 hours after starting the assay. Measurements were recorded for a total of 4.25 hours, which is when log phase growth tended to stop in previous pilot assays. The results suggest that adding methanol after a 1.5-hour delay caused growth after 4.25 hours to be nearer in OD_{620} to growth in the absence of methanol in comparison to methanol added at the beginning of the assay (**Appendix 4**). This is supported by the observation that, at 1.5 hours, growth curves of *A. hydrophila* with methanol added initially were lower in OD_{620} than *A. hydrophila* in the other three treatments that did not contain methanol at that point. Until the addition of methanol 1.5 hours into the assay, the other three treatments overlapped almost exactly. Interestingly, the addition of 2.4 µL methanol before assays seemed to cause an increase in growth of *A. hydrophila* after 2.4 hours.

In order to determine whether or not a similar effect would occur if natural frog alkaloids were added after a 1.5-hour delay, this pilot was replicated with the addition of two more treatments: 4.8 µL of alkaloids in methanol from frog GAN-2F (female frog collected from Gandoca) after a 1.5-hour delay and 4.8 µL of alkaloids in methanol from frog GAN-3F (female frog from Gandoca) after a 1.5-hour delay. These two frogs were selected for this pilot because they had the widest variation in alkaloid quantity of frogs available at that point in the experiment (414 µg vs. 45 µg per frog skin). Following the procedure from the previous pilot assay, nearly identical results were obtained with the addition of the two 4.8 µL alkaloid treatments (**Appendix 5**). Growth of *A. hydrophila* that was inoculated with methanol after a 1.5-hour delay was slightly closer in OD_{620} to that of *A. hydrophila* in the absence of methanol, and 2.4 µL methanol added prior to the assay caused a slight increase in OD_{620} after 4.25 hours. Delayed addition of 4.8 µL alkaloids in methanol from GAN-2F, which contained a significantly higher quantity of alkaloids than GAN-3F, caused OD_{620} to be lower after 4.25 hours in comparison to delayed addition of 4.8 µL methanol alone. Delayed addition of 4.8 µL alkaloids in methanol from GAN-3F resulted in OD_{620} similar to delayed addition of methanol alone, though still lower than growth of *A. hydrophila* in the absence of methanol. These results suggest that alkaloids from frogs with a relatively high quantity of alkaloids may inhibit growth, while frogs with relatively small quantities of alkaloids may not show inhibition compared to the vehicle control. Inoculation of methanol and alkaloids prior to beginning assays was ultimately selected as the most appropriate procedure since the delayed addition of treatments required removing the microplate from the microplate reader and a

45-minute period of inoculating subsamples with methanol and alkaloids during which the subsamples would grow exponentially.

Lastly, to verify that the addition of alkaloids inhibited growth after 24.5 hours compared to methanol alone, a pilot assay was conducted using triplicate subsamples of 1:10 diluted *A. hydrophila* in seven treatments: absence of methanol, 2.4 µL methanol, 4.8 µL methanol, 2.4 µL alkaloids from GAN-1F (female frog from Gandoca), 4.8 µL alkaloids from GAN-1F, 2.4 µL alkaloids from GAN-2M (male frog from Gandoca), and 4.8 µL alkaloids from GAN-2M. GAN-1F and GAN-2M were selected because they differed in alkaloid quantity, with GAN-1F (280 µg) having a lower quantity of alkaloids compared to GAN-2M (421 µg). Different frogs from the previous pilot were used in order to limit the quantity of alkaloids used from individual frogs, given the limited amount of frog alkaloids available for the experiment. The results from this pilot assay are in line with previous pilots in that 2.4 µL methanol did not inhibit growth significantly compared to untreated *A. hydrophila* (**Appendix 6**). The results also indicate that frogs with relatively high quantities of alkaloids (GAN-2M in this case) inhibit growth of *A. hydrophila* compared to methanol alone and compared to frogs with relatively low quantities of alkaloids (GAN-1F here). This is most apparent in volumes of 2.4 µL, where alkaloids from GAN-2M were visibly more inhibitory than methanol and alkaloids from GAN-1F. Although alkaloid inhibition may not be as apparent at volumes of 4.8 µL, both frogs' alkaloids resulted in OD_{620} measurements that were slightly below growth with 4.8 µL methanol alone.

Aeromonas hydrophila CFU Pilot Assays

In order to determine the number of serial dilutions required to obtain single colonies from growing 10 µL aliquots of *Aeromonas hydrophila* on TSA, pilot CFU assays were conducted following the first five pilots, including four 24.5-hour OD pilot assays and the first pilot examining mid-log addition of treatments. In the first four pilots, two subsamples of each treatment were serially diluted seven times and plated on TSA in 10 µL aliquots. In the fifth pilot, all three subsamples used were serially diluted seven times and then plated on TSA in 10 µL aliquots. The overall conclusion from serial dilution pilots is that a minimum of six serial dilutions is generally required to observe single colonies below 30 CFUs per aliquot. Additionally, greater volumes of methanol appear to result in lower CFU counts, suggesting that viability of *A. hydrophila* cells decreases with exposure to methanol, which is consistent with results from pilot OD assays. Regarding CFU counts for the fifth pilot, which compared initial addition and mid-log addition of 2.4 µL and 4.8 µL methanol, the average CFU count parallels trends in the OD assay almost exactly: mid-log addition of methanol resulted in growth comparable to growth in the absence of methanol, while initial addition of 2.4 µL methanol resulted in increased growth and initial addition of 4.8 µL methanol resulted in decreased growth (**Appendix 4 and 7**).

Klebsiella pneumoniae OD Pilot Assays

Three replicated pilot assays comparing growth curves of 1:5, 1:10, and 1:50 dilutions in the absence of methanol, with 2.4 µL methanol, and with 4.8 µL methanol were carried out to determine the optimal dilution of *K. pneumoniae* to use in OD assays with

alkaloids. Similarly to previous pilot OD assays with *A. hydrophila*, eight 200 µL subsamples for each treatment were plated in a microplate and OD_{620} was measured at 15-minute intervals over 24.5 hours. All three pilot assays produced nearly identical growth curves for each treatment at each dilution (**Appendix 8**). Similar to pilot results with *A. hydrophila*, the 1:5 dilution treatments began and ended with the highest OD_{620}, the 1:10 dilution began and ended intermediately at OD_{620}, and the 1:50 dilution began and ended with the lowest OD_{620} values. Once again, a dose-response relationship was observed with methanol treatments, where the addition of 4.8 µL methanol reduced OD_{620} more than 2.4 µL methanol after 24.5 hours. At all dilutions, the addition of 2.4 µL methanol appears to reduce OD_{620} compared to treatments with no methanol added, which was not always apparent with *A. hydrophila*. Growth curves of the 1:50 dilution appeared to start just prior to log phase, which made it the least ideal for growth inhibition assays of the three dilutions. Between the 1:5 and 1:10 dilutions, the 1:5 dilution was selected for use in assays with alkaloids on the grounds that its growth curve after 24.5 hours was nearer to leveling off (stationary phase) than the 1:10 dilution. Pilot CFU assays also played a role in selecting the 1:10 dilution for alkaloid assays. In practice, using either of the 1:5 or 1:10 dilutions did not make a difference in terms of growth inhibition results.

Klebsiella pneumoniae CFU Pilot Assays

Pilot CFU assays were carried out immediately following pilot OD assays for *K. pneumoniae*, and similar trends were observed to those in *A. hydrophila* pilot CFU assays. CFUs from 1:5 dilutions consistently required seven serial dilutions in the

absence of methanol and with the addition of both 2.4 µL and 4.8 µL methanol. The 1:10 dilution yielded CFUs below 30 in number only when methanol was added, but not in every case, and the 1:50 dilution showed considerable variation around which serial dilution yielded CFUs under 30 in number. Therefore, the 1:5 dilution was selected as most suitable for growth inhibition assays because the number of required serial dilutions was consistent and most similar to those conducted in *A. hydrophila* assays. Moreover, since the magnitude of difference between serial dilutions was factored into the original cell density equations used to report CFU assay results, using the 1:5 resulted in the least potential variation in original cell density.

Batrachochytrium dendrobatidis (Chytrid Fungus) Culture and Pilots

Culture and Maintenance of Batrachochytrium dendrobatidis

Batrachochytrium dendrobatidis (JEL 197, type strain isolated from an infected green and black poison frog (*Dendrobates auratus*) (Longcore *et al*., 1999)) was received from Alessandro Catenazzi (Southern Illinois University, Carbondale) on 1% tryptone agar. Main stock cultures were grown in 1% tryptone broth (Fisher BioReagents, Thermo Fisher Scientific, Waltham, MA) at 20°C – 22°C for ten days and then stored at 4°C. Stock cultures (500 mL) were passaged monthly.

For pilot assays, working stocks were produced by growing aliquots of stock *Bd* cultures in 1% tryptone broth in 50 mL conical tubes for under one week to ensure that cells were in an active phase of growth. When isolating zoospores from zoosporangia, 2 – 3 mL of working stock broth cultures were added to 1% tryptone agar in petri dishes (Fisherbrand, Thermo Fisher Scientific, Waltham, MA). Inoculated dishes were left open

in the laminar flow hood for 45 – 60 minutes, or until the broth had almost entirely evaporated. Dishes were then sealed with Parafilm® M (American National Can, Chicago, IL) and incubated at 20°C – 22°C for 3 – 7 days.

Active zoospores were identified by inverting the dishes on a compound microscope (Olympus CX21, Olympus, Center Valley, PA) and examining with the 10X objective. Zoospores were harvested by flooding 3 – 7 day old inoculated dishes with 2 – 3 mL sterile 1% tryptone broth or deionized water for 30 minutes. The solution containing active zoospores was then passed over sterile nylon mesh filters (20 µm) to remove zoosporangia. *Bd* zoospores were intended to be assayed as opposed to a solution containing both zoospores and mature zoosporangia, because zoospores are the infectious stage of *Bd* (Berger *et al*. 2005) and would be the first to come into contact with alkaloid-based defenses of poison frogs. Therefore, alkaloid-zoospore assays are the most appropriate first step towards studying the ecological interactions of *Bd* with alkaloids. Zoospores were counted using a hemacytometer (Reichert-Jung Bright-Line, Hausser Scientific, Horsham, PA).

The first pilot for *Bd* sought to determine a typical growth curve of the unfiltered 1% tryptone broth culture in the absence of methanol, with 2.4 µL methanol, and with 4.8 µL methanol. Similarly to pilot OD assays with bacteria, 200 µL subsamples of 1:5 diluted *Bd* from a main stock were plated in a microplate with eight subsamples per treatment: two control samples (i.e., two columns with eight replicates each) of *Bd* in the absence of methanol, one sample of *Bd* inoculated with 2.4 µL methanol, and one sample of *Bd* inoculated with 4.8 µL methanol. Optical density measurement filters on the Fisher Scientific Multiskan FC microplate reader allowed measurement at 405 nm, 450 nm, 550

nm, and 620 nm; the lowest (OD_{405}) and highest (OD_{620}) wavelengths were used to measure growth. Due to the prolonged growth period of *Bd*, this assay ran for seven days; since the microplate reader is limited to 100 measurements per assay, seven consecutive runs of 24-hour programs that measured OD_{405} and OD_{620} at 15-minute intervals were carried out. Based on the optical density measurements over seven days (**Appendix 9**), growth of *Bd* at this dilution is clearly very slow, and would undoubtedly have continued to increase past the end of this assay (as shown in the second *Bd* pilot). Methanol also clearly inhibits the growth of *Bd*, and there is an evident dose-response relationship between inoculation with 2.4 µL and 4.8 µL methanol. Additionally, there are regular four- to six-hour fluctuations in OD throughout the assay, although this does not appear to be typical in *Bd* growth (Alessandro Catenazzi, pers. comm.). Finally, the growth curves of replicate treatments measured at 405 nm and 620 nm showed identical growth patterns, but OD measurements at lower wavelengths seemed to distinguish greater differences between treatments. As a result, 405 nm was selected as the ideal wavelength to use in OD assays with *Bd*. However, most antimicrobial peptide assays with *Bd* use a 492 nm wavelength filter for similar OD assays, which were unavailable, so it may be worthwhile to locate a 492 nm filter for the Fisher Scientific Multiskan FC microplate reader.

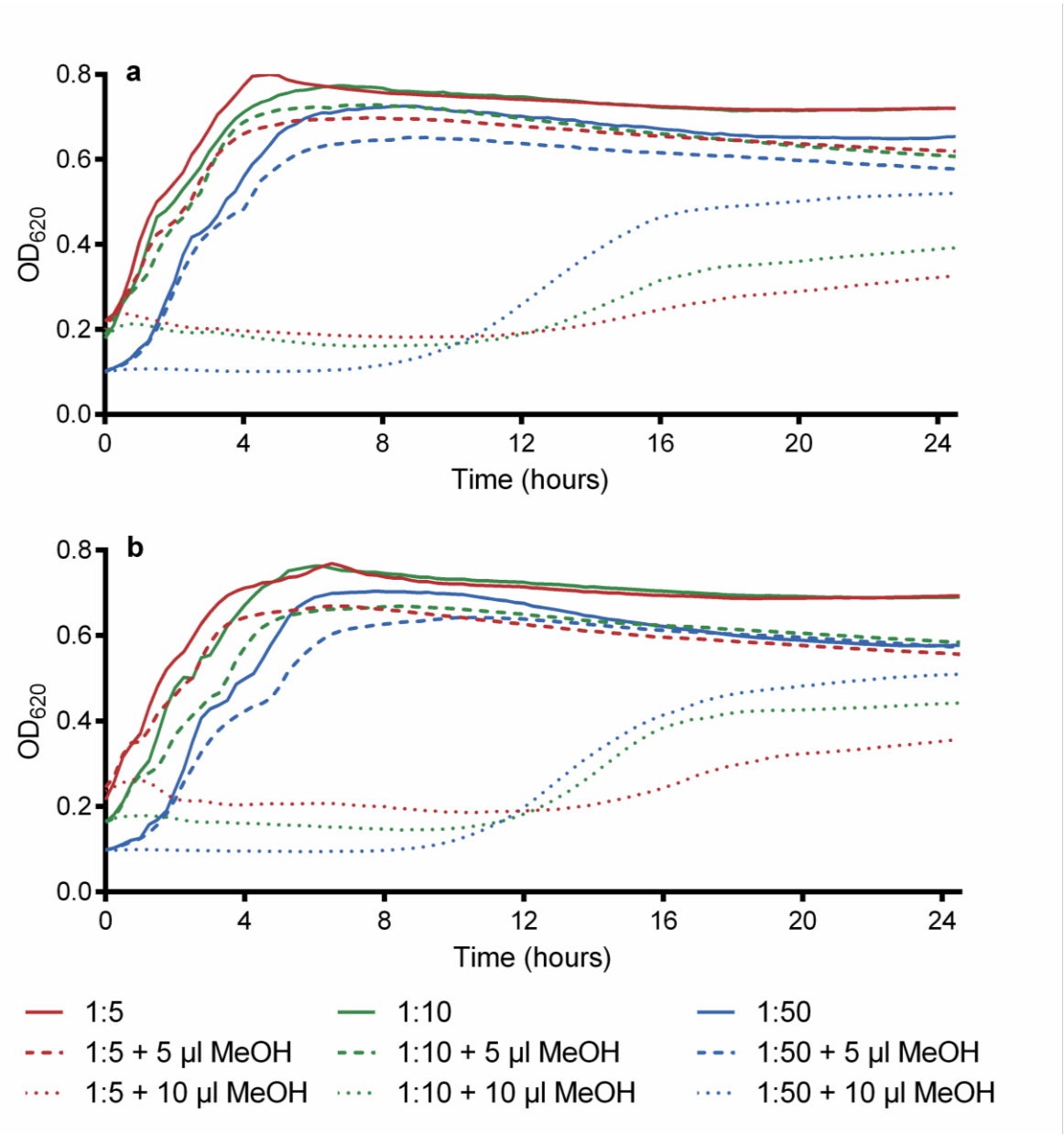
In order to characterize the growth of *Bd* after one week, the treatments for the first pilot experiment was replicated and *Bd* growth was measured over the course of 14 days. The program measured OD_{405} at 4-hour intervals. The results of this second pilot showed a very similar growth pattern in which growth starts to become noticeable between 72 and 96 hours (**Appendix 10**) suggest that *Bd* growth curves can be relatively

consistent. However, the initial OD_{405} was slightly reduced compared to initial OD_{405} in the first pilot (about 0.12 OD compared to 0.13 OD), and this difference appears to be retained in similar proportion at the end of the assay. For example, in this second pilot, the OD_{405} after 168 hours was about 0.19 for non-methanol treatments and 0.25 at the end of the second pilot (i.e., after 336 hours), whereas OD_{405} for the same treatments in the first pilot after 168 hours was nearly 0.23. Clearly, small differences in OD at the outset of *Bd* assays can result in substantial differences in growth after one and two weeks, which has the potential to confound comparisons between different assays. Moreover, since *Bd* grows so slowly, maintaining a consistent starting OD is likely the primary methodological challenge, especially if using only zoospores.

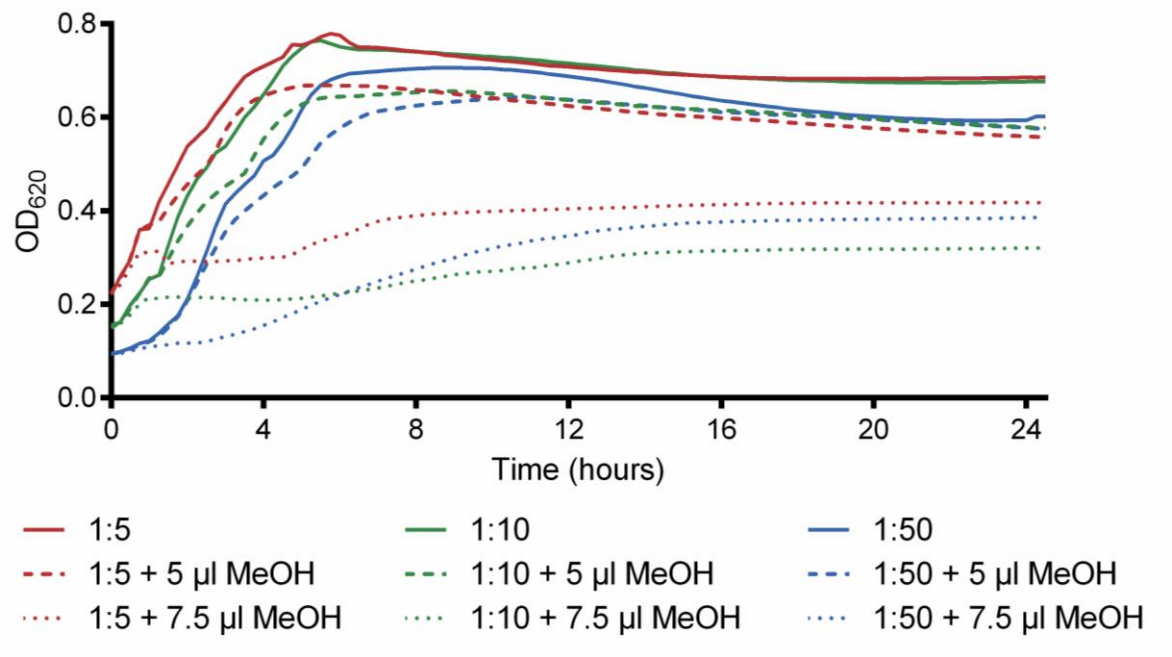
Bd zoospores were successfully isolated from cultured zoosporangia and counted on a hemacytometer, although no growth curves were generated from exploratory experiments. A variety of methodological difficulties were encountered throughout pilot experimentation with *Bd*. For example, the culture media (1% tryptone) was determined to react with iodine used in killing and staining of motile zoospores, which is highly useful in counting zoospores to estimate the quantity of zoospores per mL solution. The reaction between tryptone and iodine produced dense clouds of red-brown, rapidly-vibrating objects that were several times larger than zoospores, but also gradually reduced their movement and settled onto the hemacytometer surface as if they had been stained and killed. This reaction did not occur between iodine and sterile water, however, which would likely be the best solution to count zoospores in. Alternatively, there are a variety of other dyes available (e.g., trypan blue, although only dead zoospores absorb the

dye (McMahon and Rohr, 2014)) that may be worthwhile to invest in for counting zoospores.

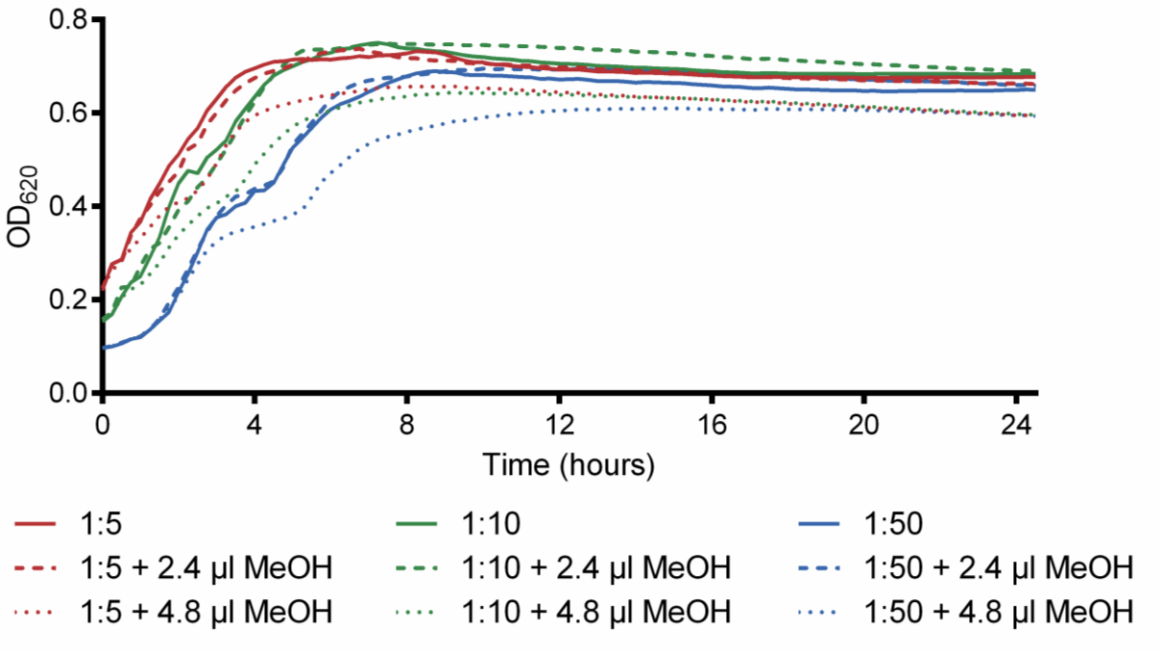
Zoospores were rarely isolated in densities exceeding 5×10^5 zoospores/mL, which is near the lower limit for accurate counting on the hemacytometer. To acquire higher zoospore densities, denser mats of *Bd* zoosporangia on multiple agar plates (but no more than 20 should be fit in the hood, spread out with lids off) must be cultured. Accordingly, liquid cultures of *Bd* must also be dense but in an active phase of growth; it may be useful to plate the settled zoosporangia from liquid cultures, as opposed to agitating the culture before removing zoosporangia. Similarly, working cultures likely do not need to be diluted much (e.g., 1:10 in first two *Bd* pilot experiments).



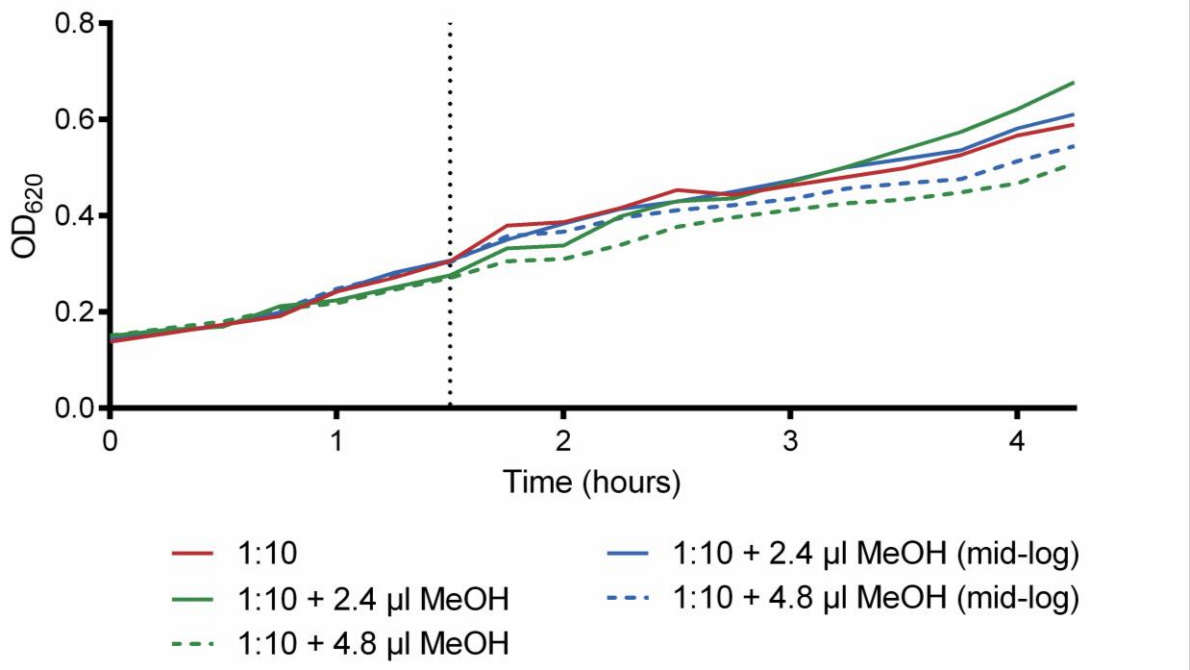
Appendix 1. Growth curves of *Aeromonas hydrophila* from (**a**) pilot experiment 1 and (**b**) pilot experiment 2. Cultures diluted to 1:5, 1:10, and 1:50 had 0 µL, 5 µL, or 10 µL methanol added. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



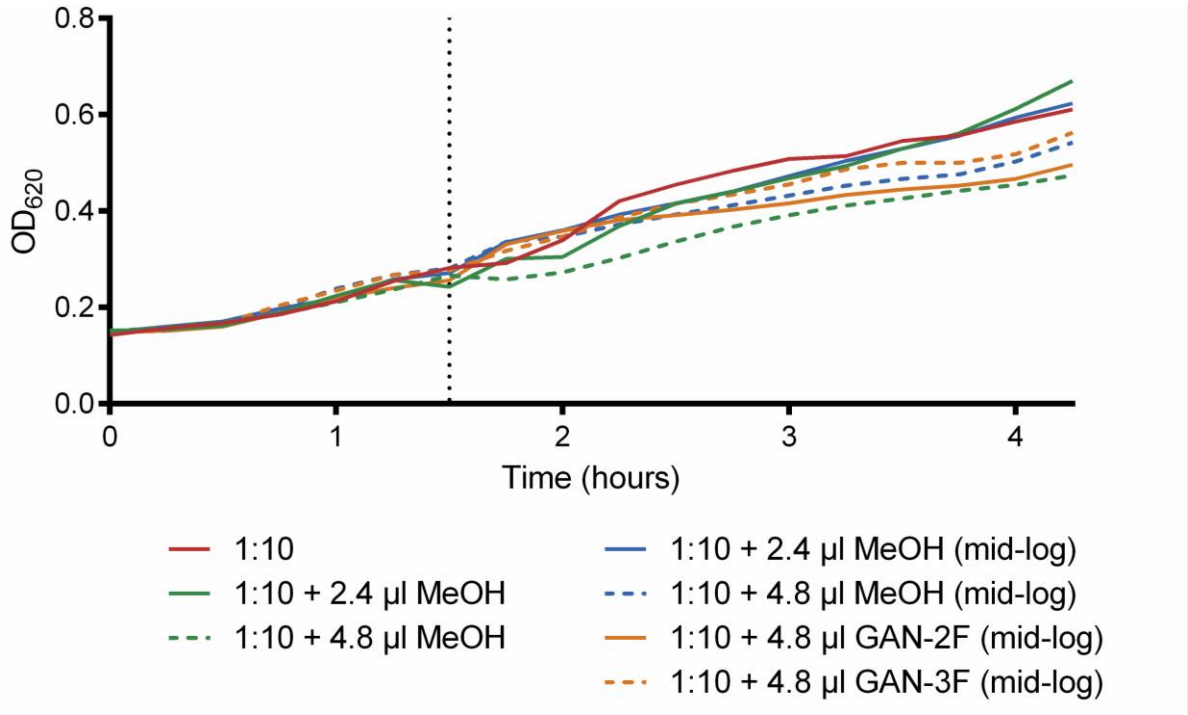
Appendix 2. Growth curves of *Aeromonas hydrophila* from pilot experiment 3. Cultures diluted to 1:5, 1:10, and 1:50 had 0 µL, 5 µL, or 7.5 µL methanol added. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



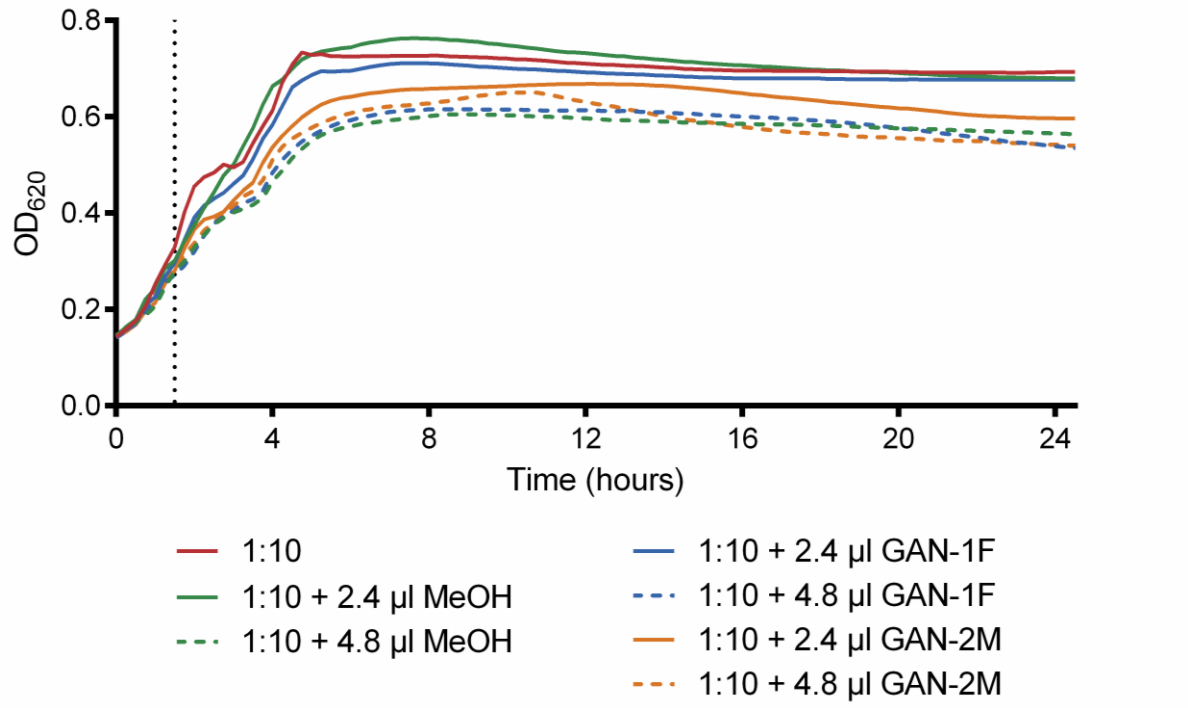
Appendix 3. Growth curves of *Aeromonas hydrophila* from pilot experiment 4. Cultures diluted to 1:5, 1:10, and 1:50 had 0 µL, 2.4 µL, or 4.8 µL methanol added. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



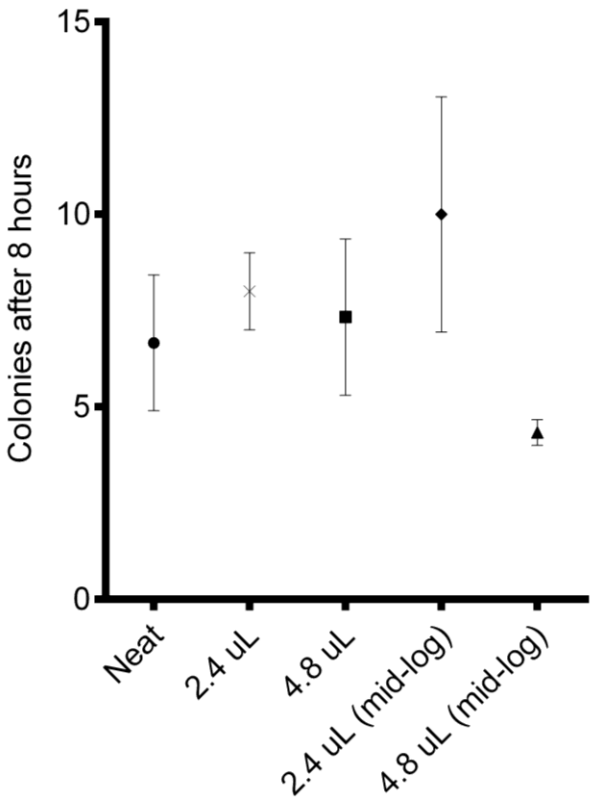
Appendix 4. Growth curves of *Aeromonas hydrophila* from pilot experiment 5. Cultures diluted to 1:10 had 0 µL, 2.4 µL, or 4.8 µL methanol added initially or after 1.5 hours of incubation ("mid-log"). OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours. The dotted line represents the point at which mid-log treatments were added.



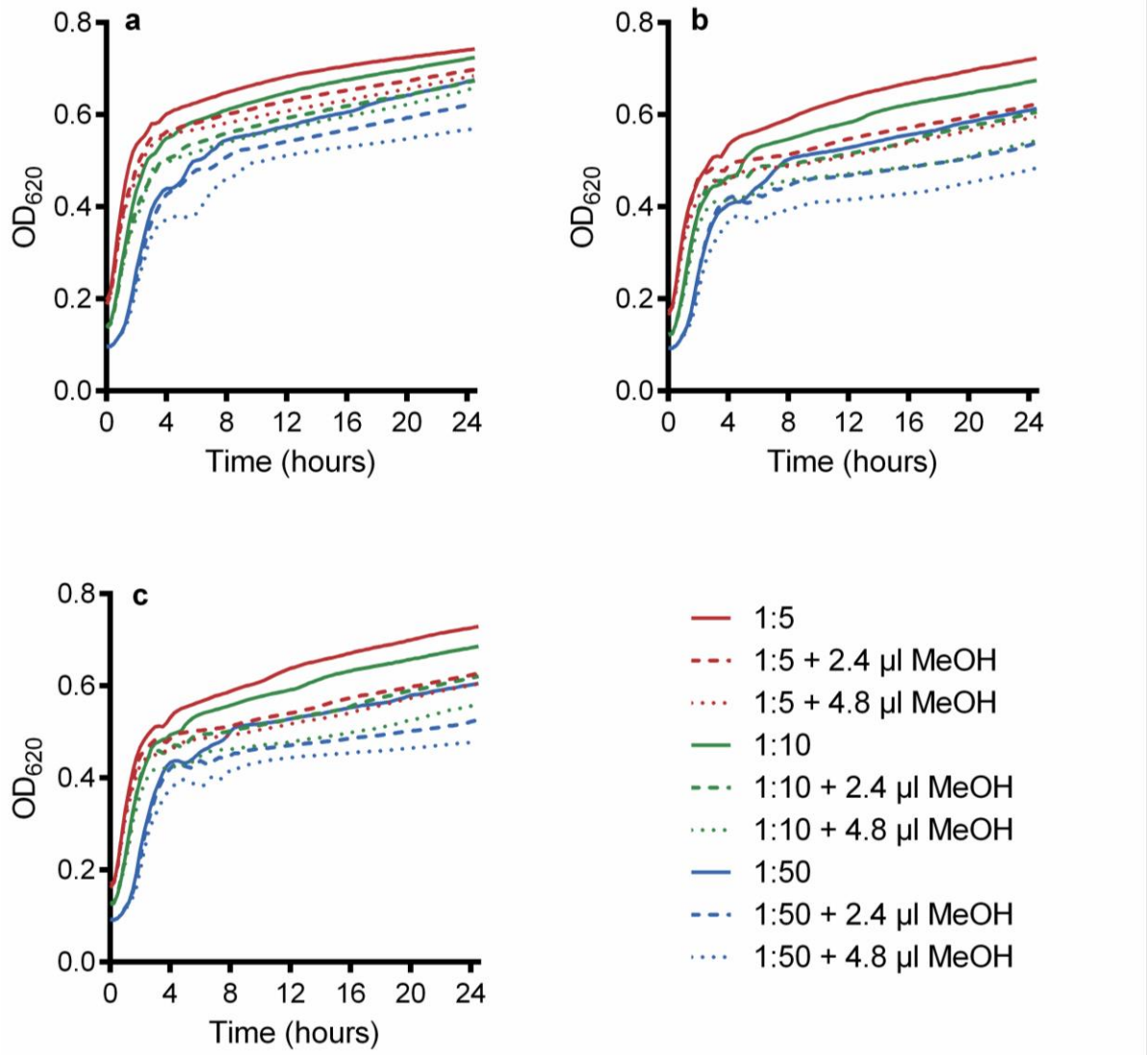
Appendix 5. Growth curves of *Aeromonas hydrophila* from pilot experiment 6. Cultures diluted to 1:10 had 0 µL, 2.4 µL, or 4.8 µL methanol or *Oophaga pumilio* alkaloids added initially or after 1.5 hours of incubation ("mid-log"). OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours. The dotted line represents the point at which mid-log treatments were added.



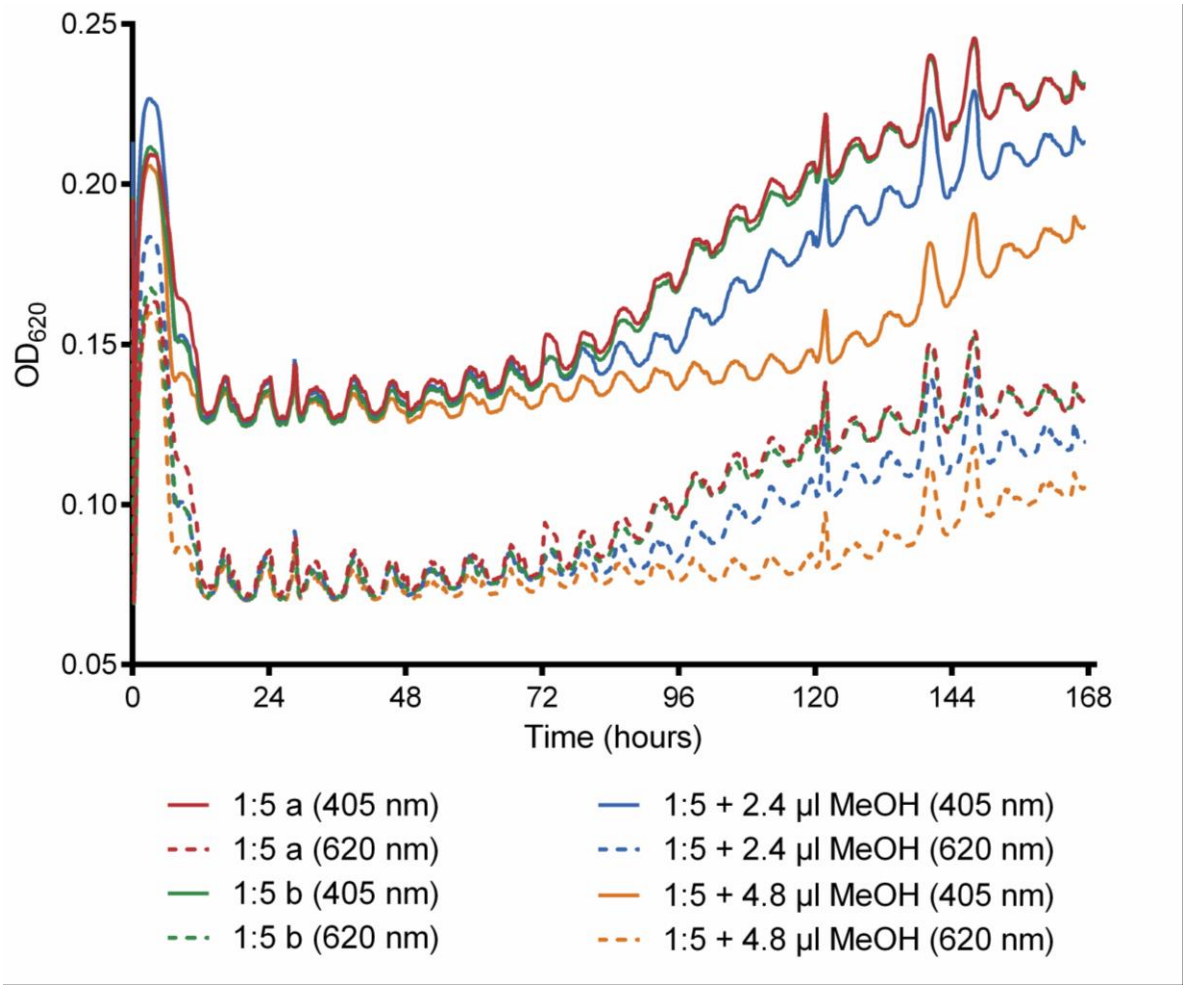
Appendix 6. Growth curves of *Aeromonas hydrophila* from pilot experiment 7. Cultures diluted to 1:10 had 0 µL, 2.4 µL, or 4.8 µL methanol or *Oophaga pumilio* alkaloids added. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours. The dotted line represents the point at which mid-log treatments were added.



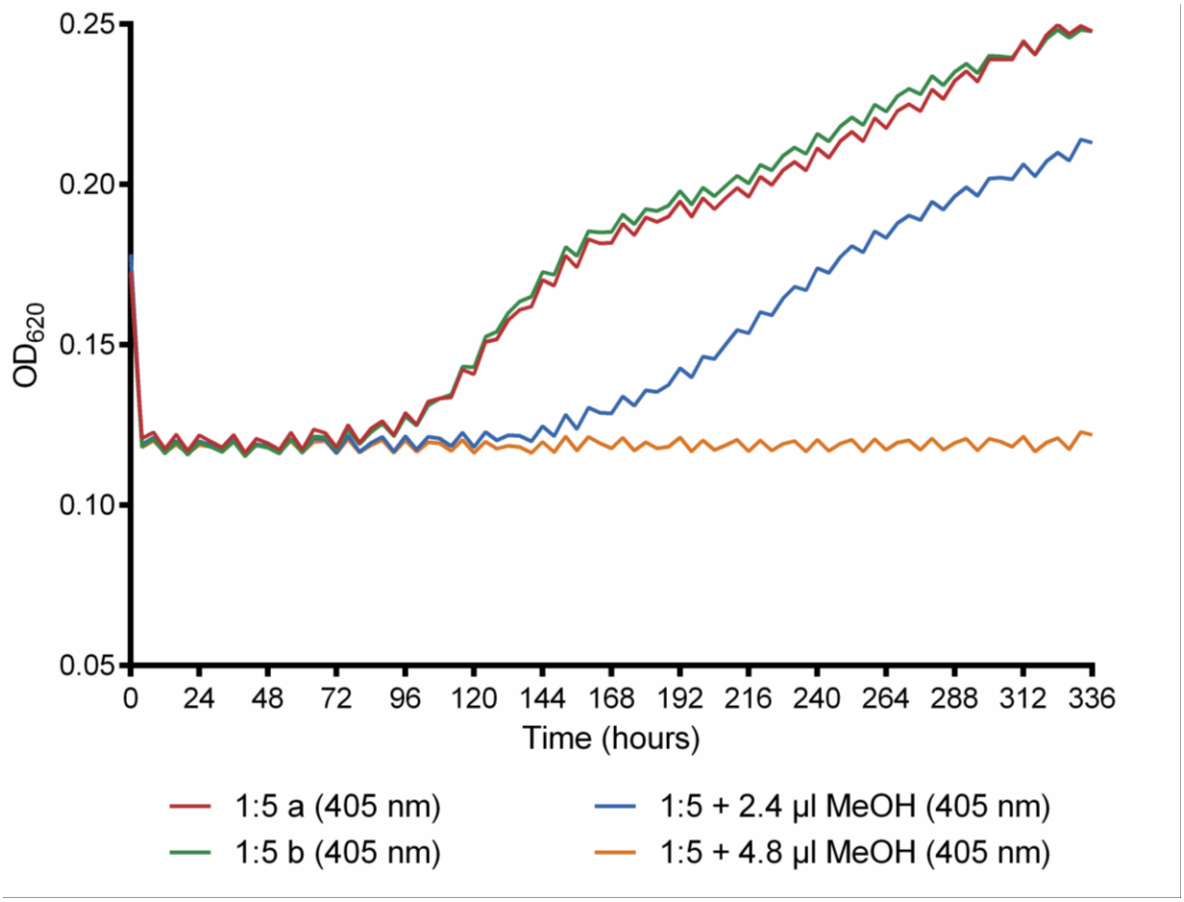
Appendix 7. Mean colonies formed per 10 µL aliquot from treatments within *Aeromonas hydrophila* pilot experiment 5 (1:10 diluted initial culture). Error bars represent ± 1 standard error of the mean, based on three replicates per treatment.



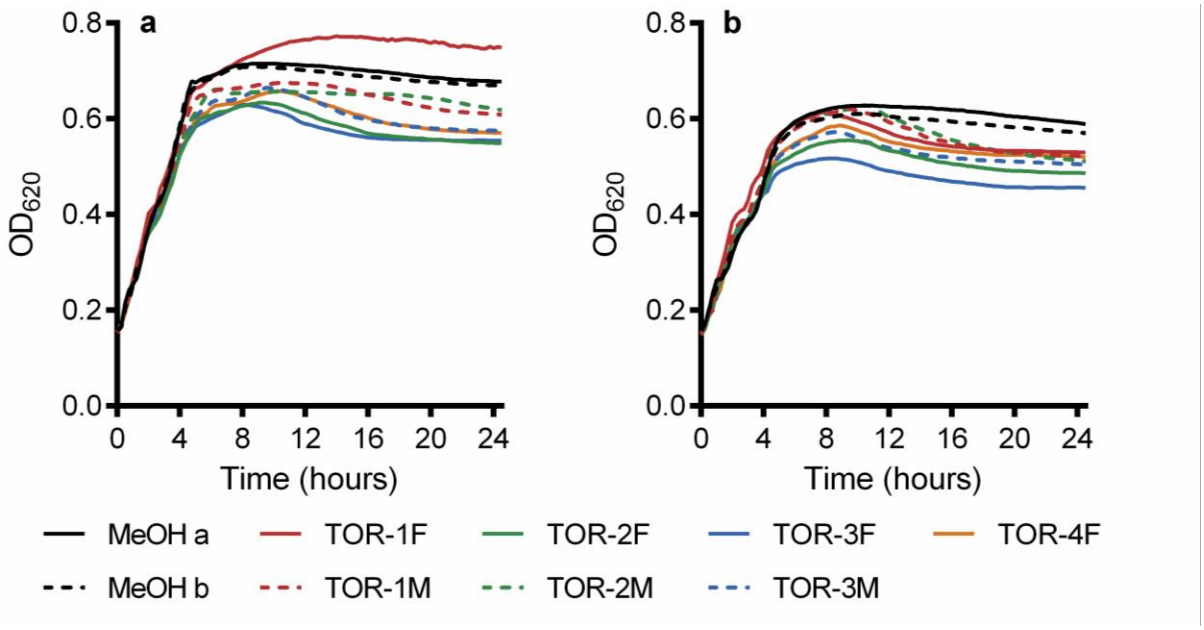
Appendix 8. Growth curves of *Klebsiella pneumoniae* from (**a**) pilot experiment 1, (**b**) pilot experiment 2, and (**c**) pilot experiment 3. Cultures diluted to 1:10 had 0 µL, 2.4 µL, or 4.8 µL methanol added initially. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



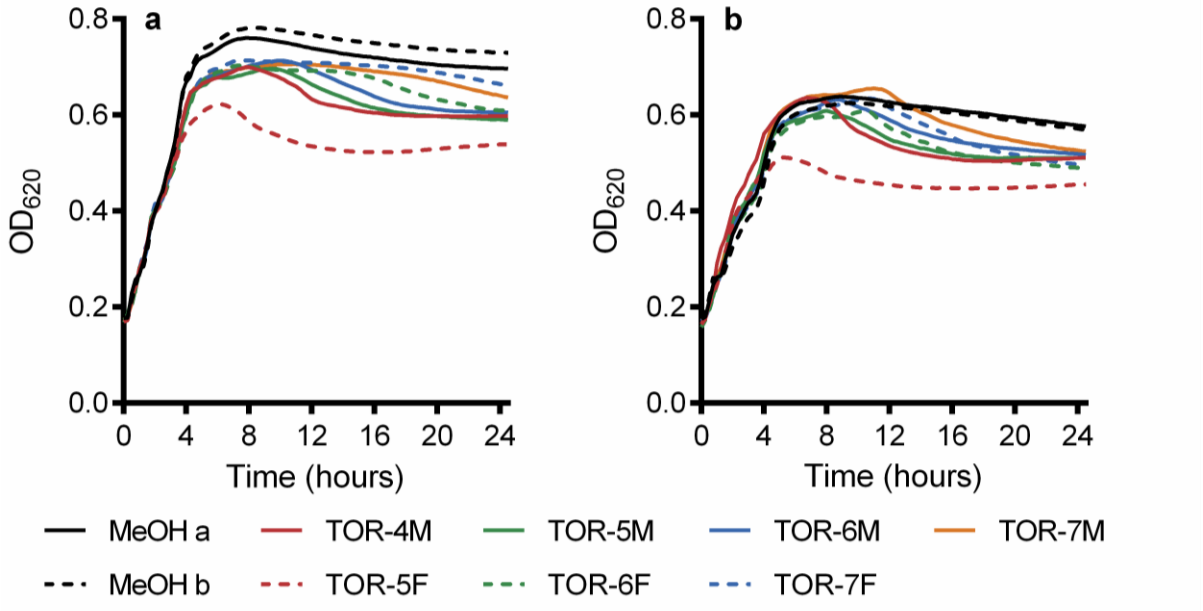
Appendix 9. Growth curves of *Batrachochytrium dendrobatidis* from pilot experiment 1. Cultures diluted to 1:5 had 0 µL, 2.4 µL, or 4.8 µL methanol added initially. OD_{405} and OD_{620} measurements were recorded at 1-hour intervals over the course of 7 days.



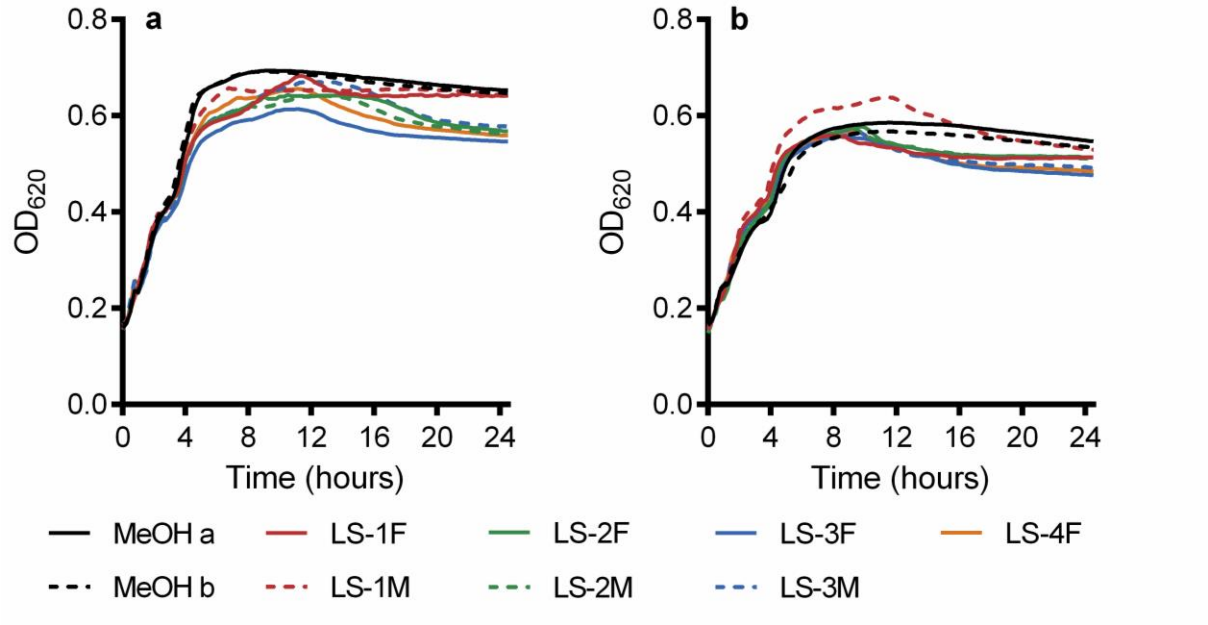
Appendix 10. Growth curves of *Batrachochytrium dendrobatidis* from pilot experiment 2. Cultures diluted to 1:5 had 0 µL, 2.4 µL, or 4.8 µL methanol added initially. OD_{405} measurements were recorded at 4-hour intervals over the course of 14 days.



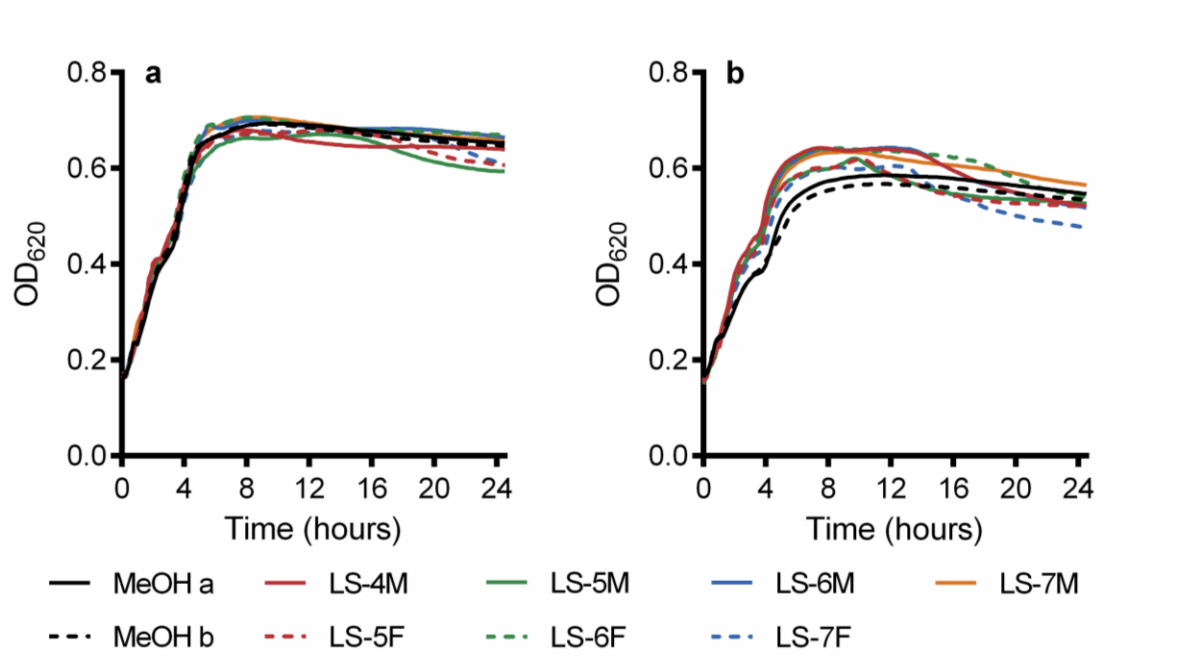
Appendix 11. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Tortuguero *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



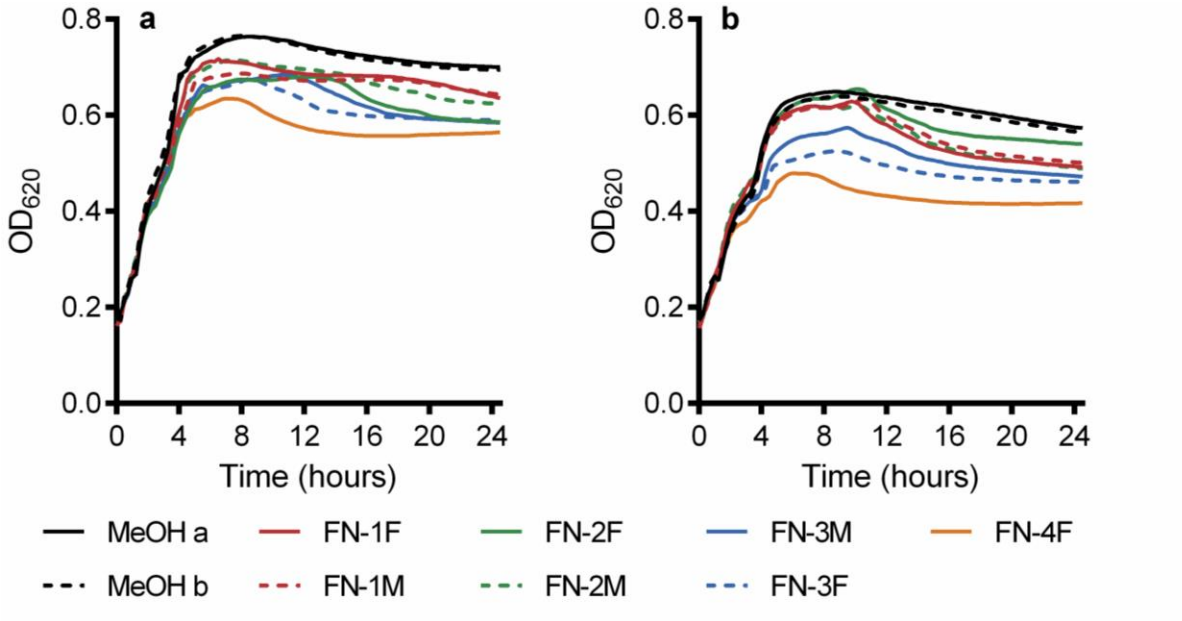
Appendix 12. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Tortuguero *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



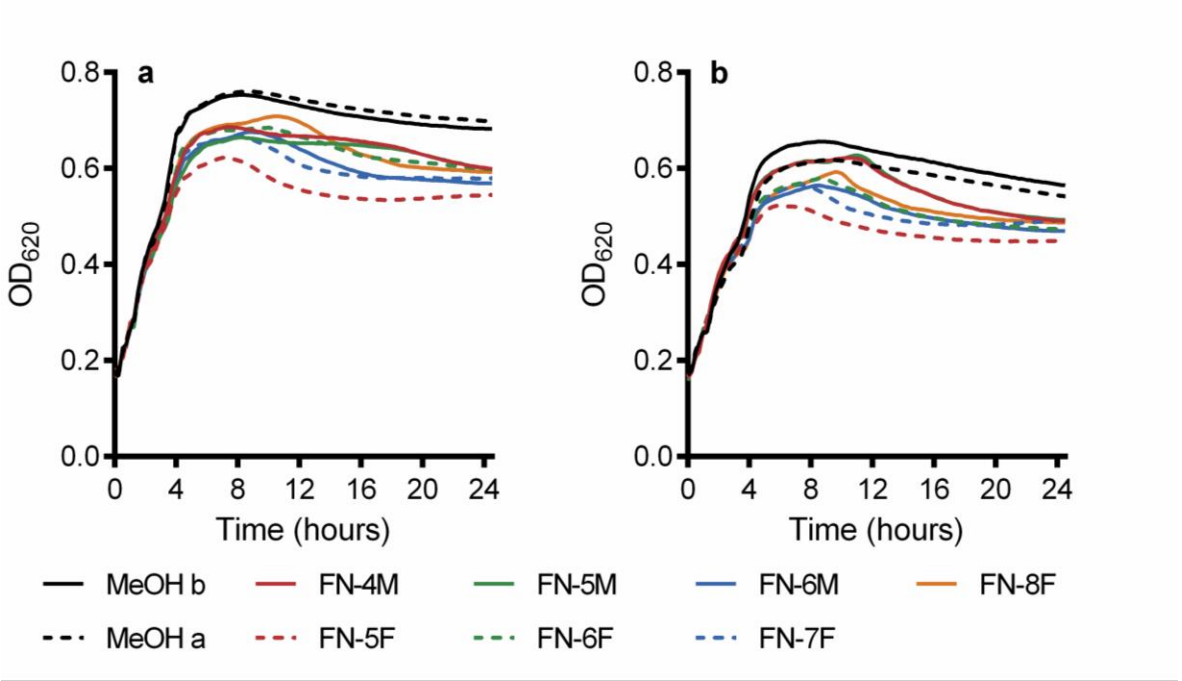
Appendix 13. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or La Selva *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



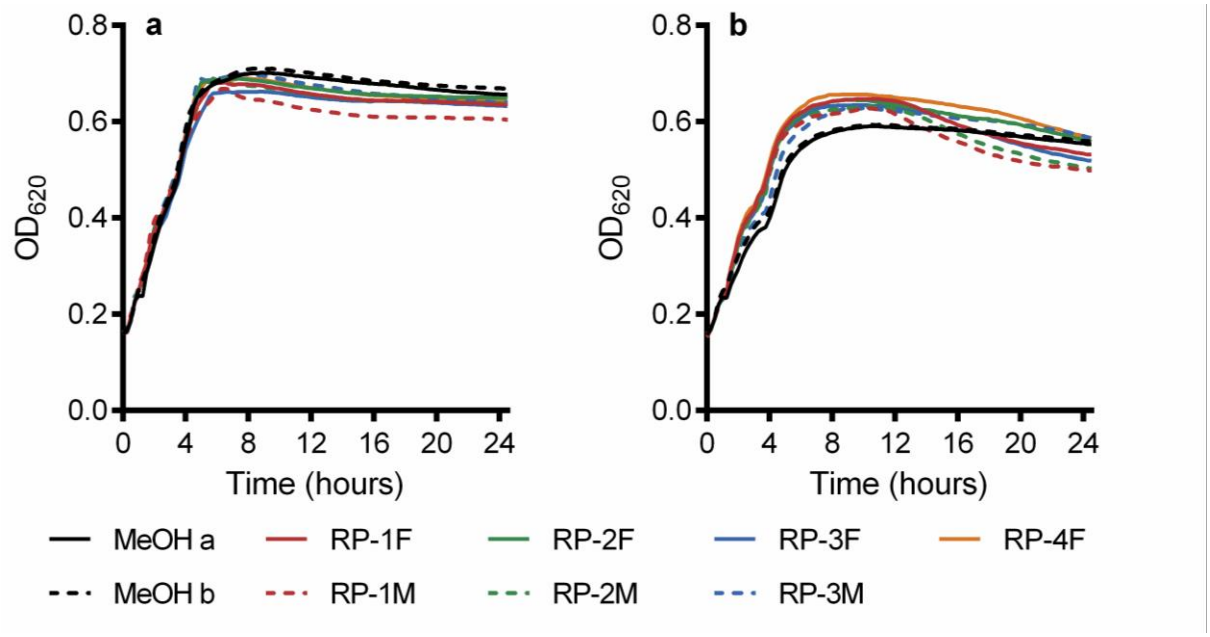
Appendix 14. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or La Selva *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



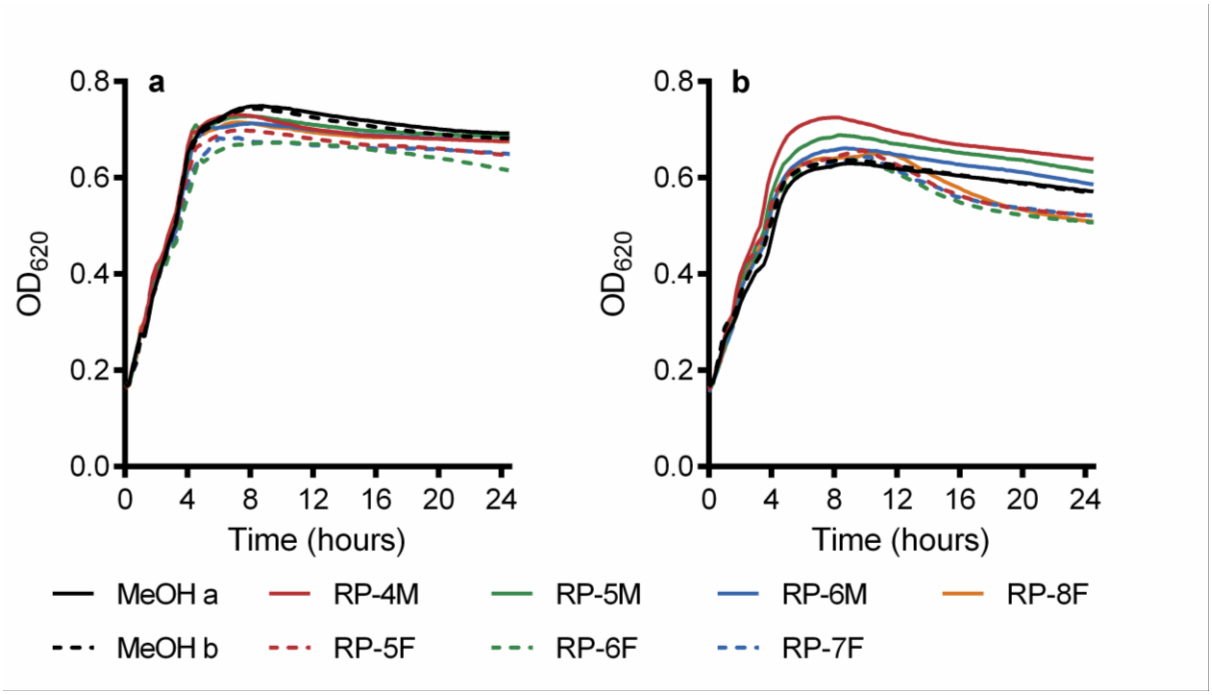
Appendix 15. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Finca los Nacientes *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



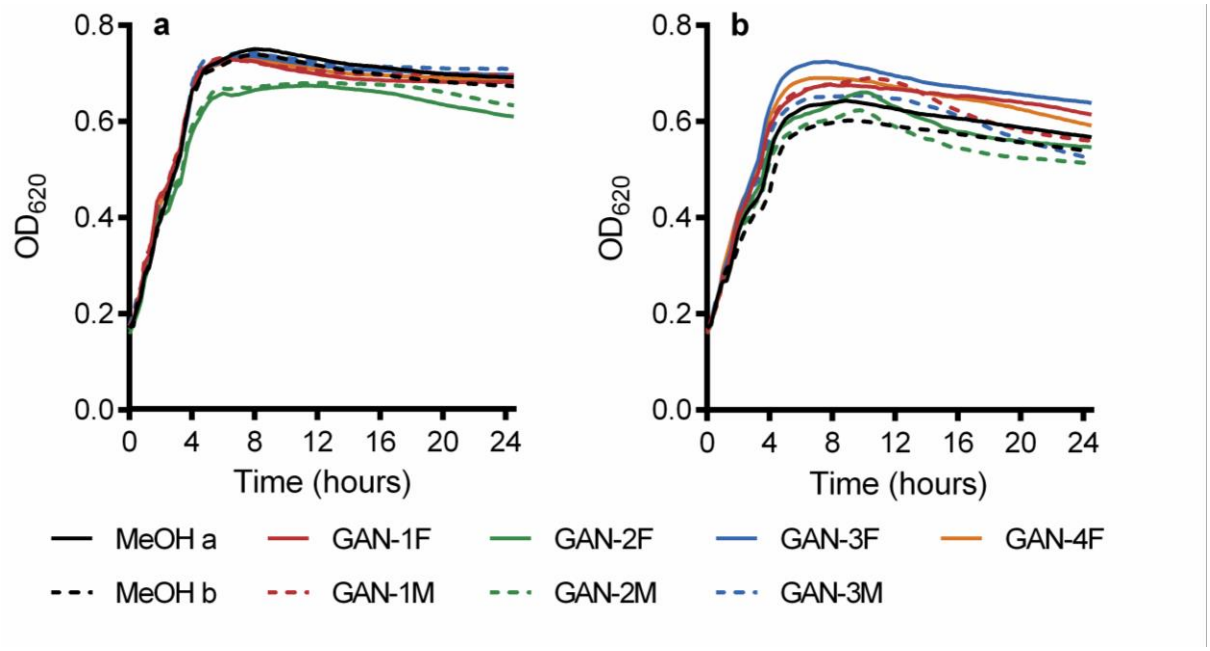
Appendix 16. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Finca los Nacientes *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



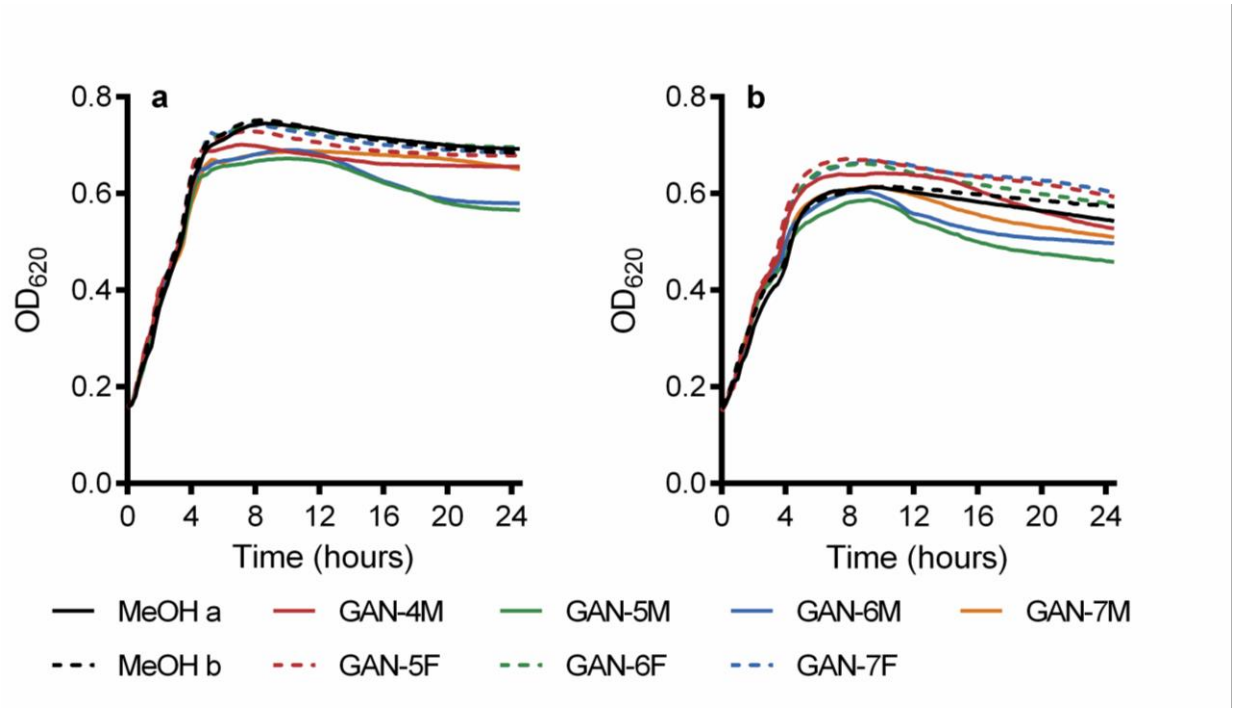
Appendix 17. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Río Palmas *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



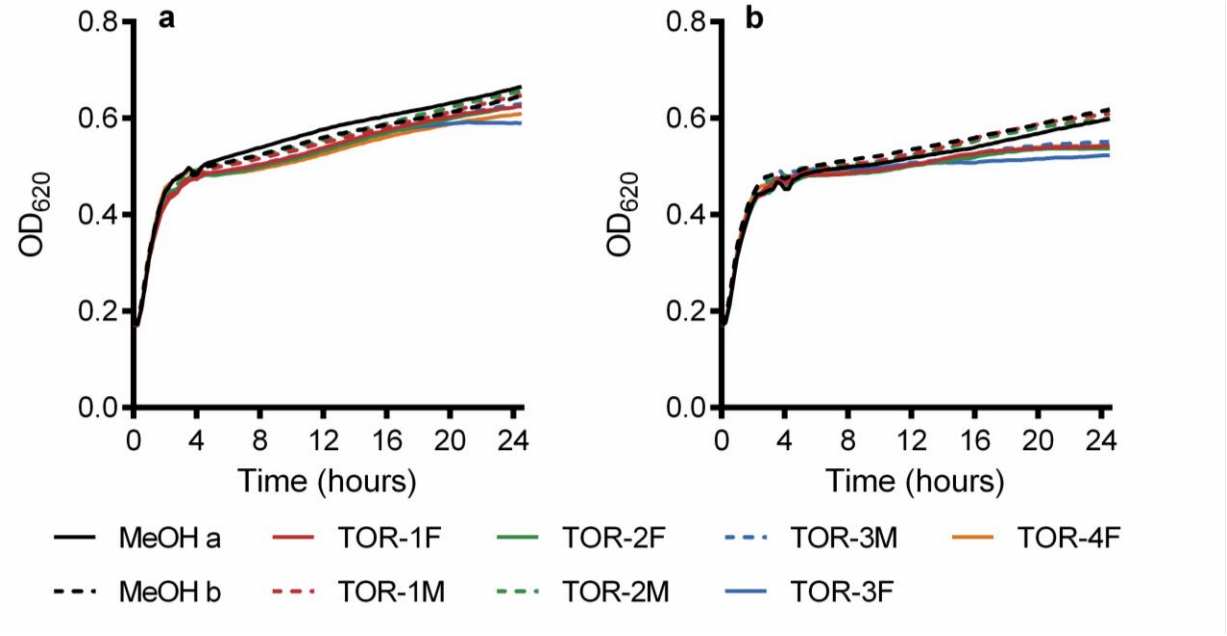
Appendix 18. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Río Palmas *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



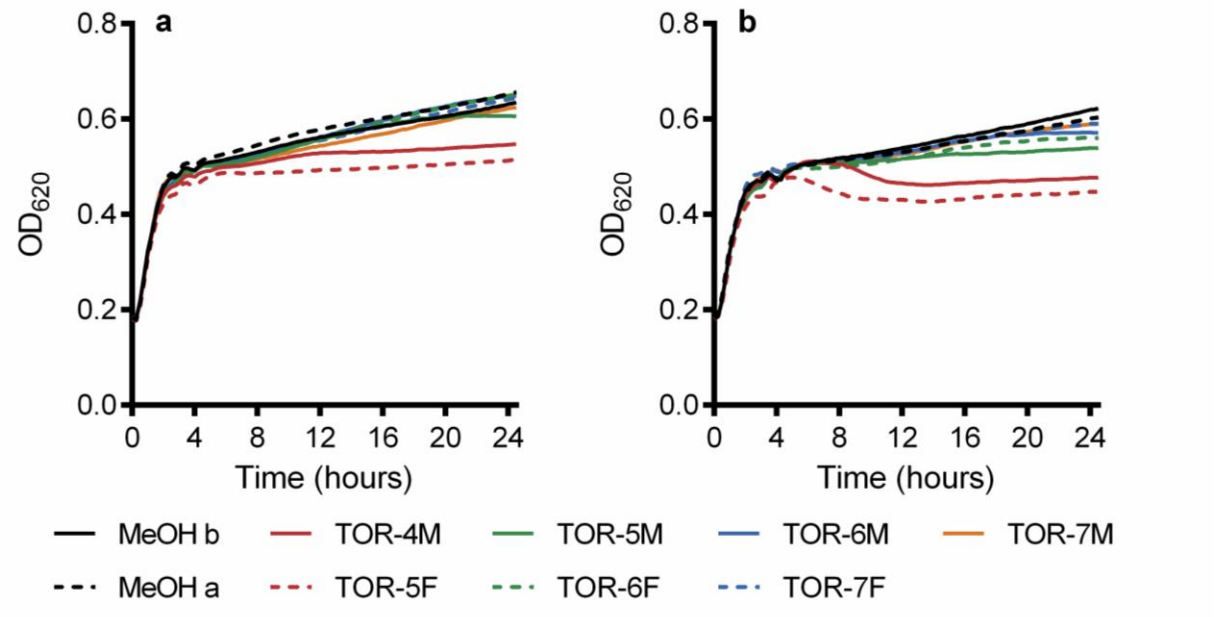
Appendix 19. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Gandoca *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



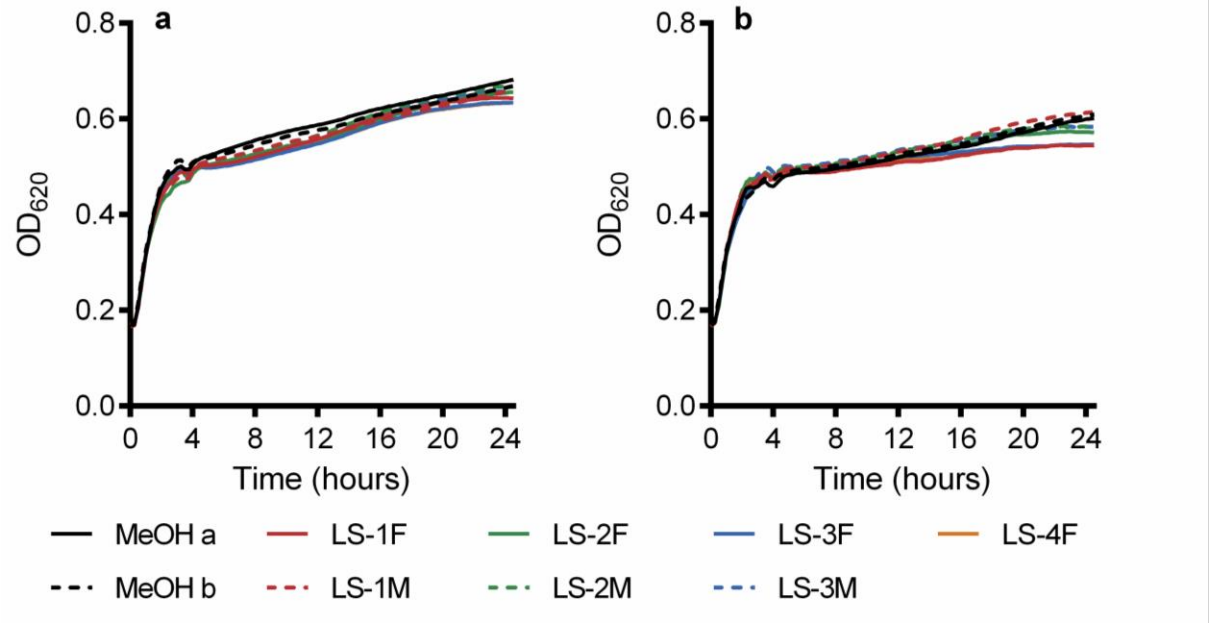
Appendix 20. Growth curves of *Aeromonas hydrophila* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Gandoca *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



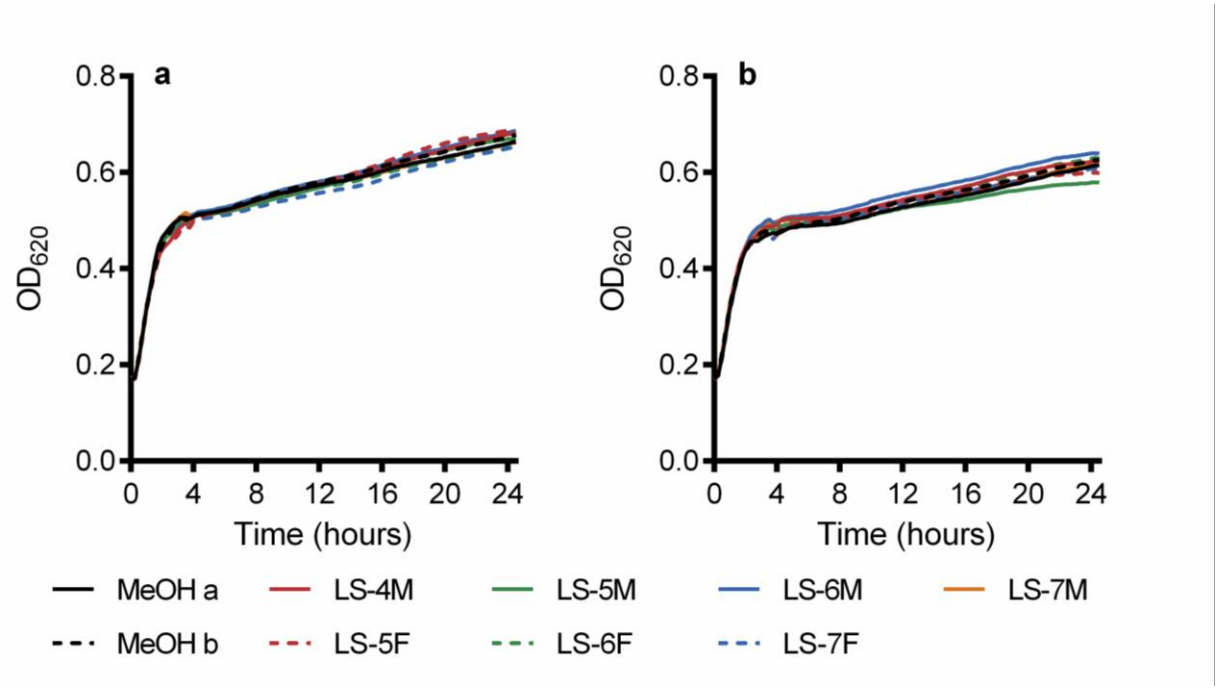
Appendix 21. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Tortuguero *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



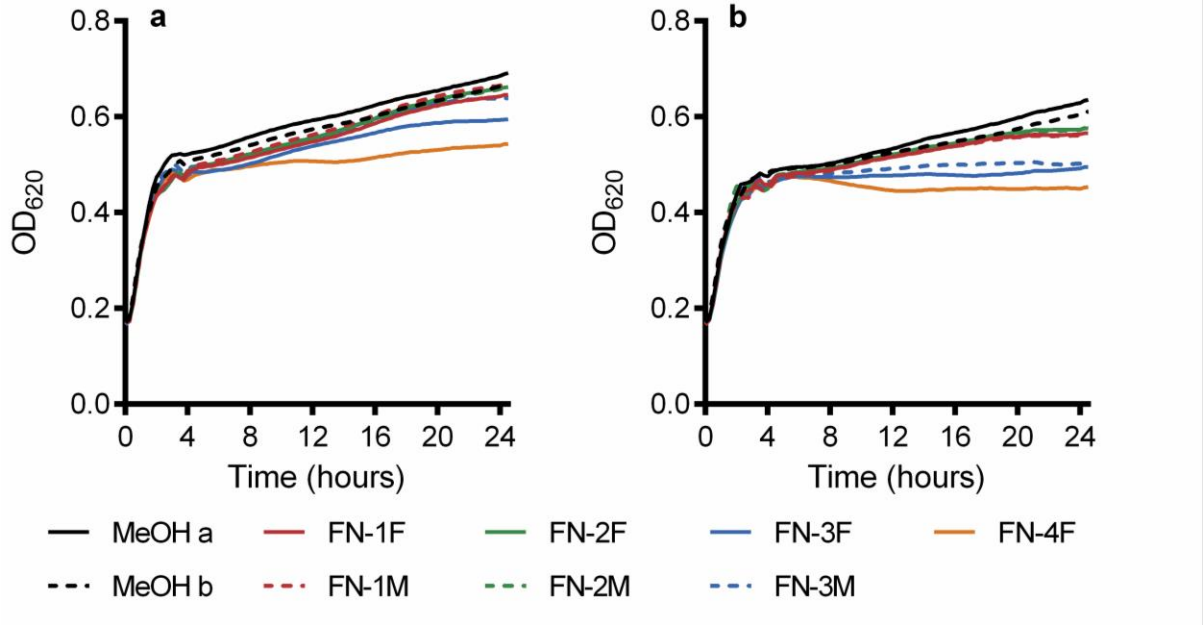
Appendix 22. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Tortuguero *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



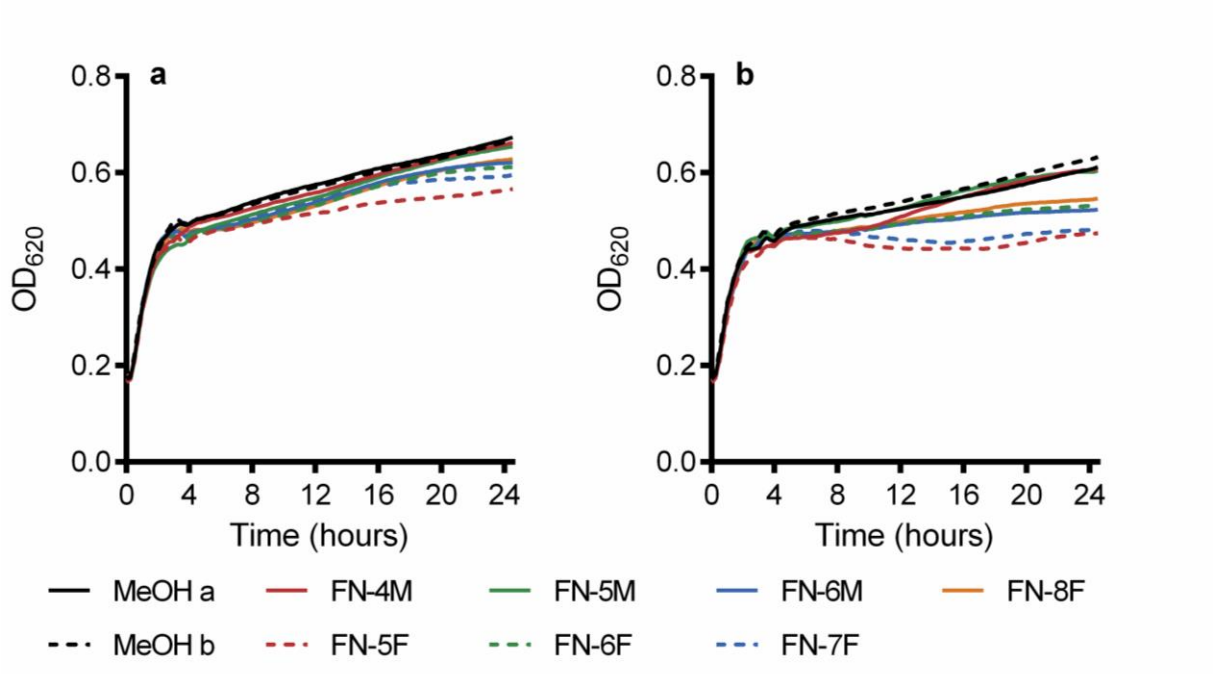
Appendix 23. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or La Selva *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



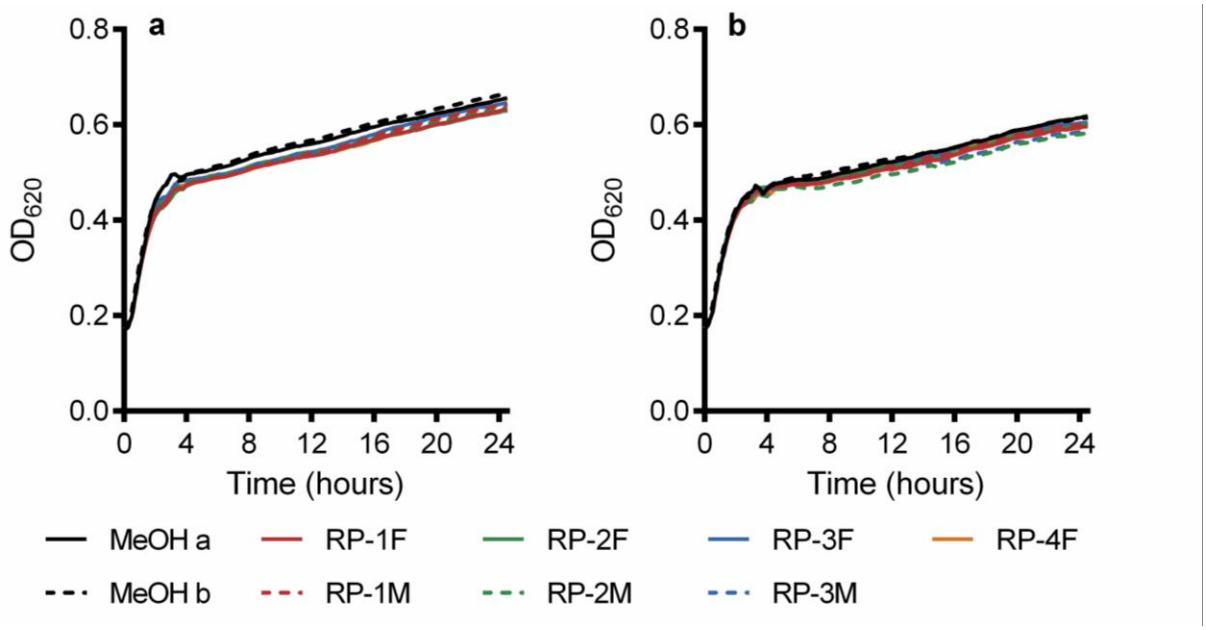
Appendix 24. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or La Selva *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



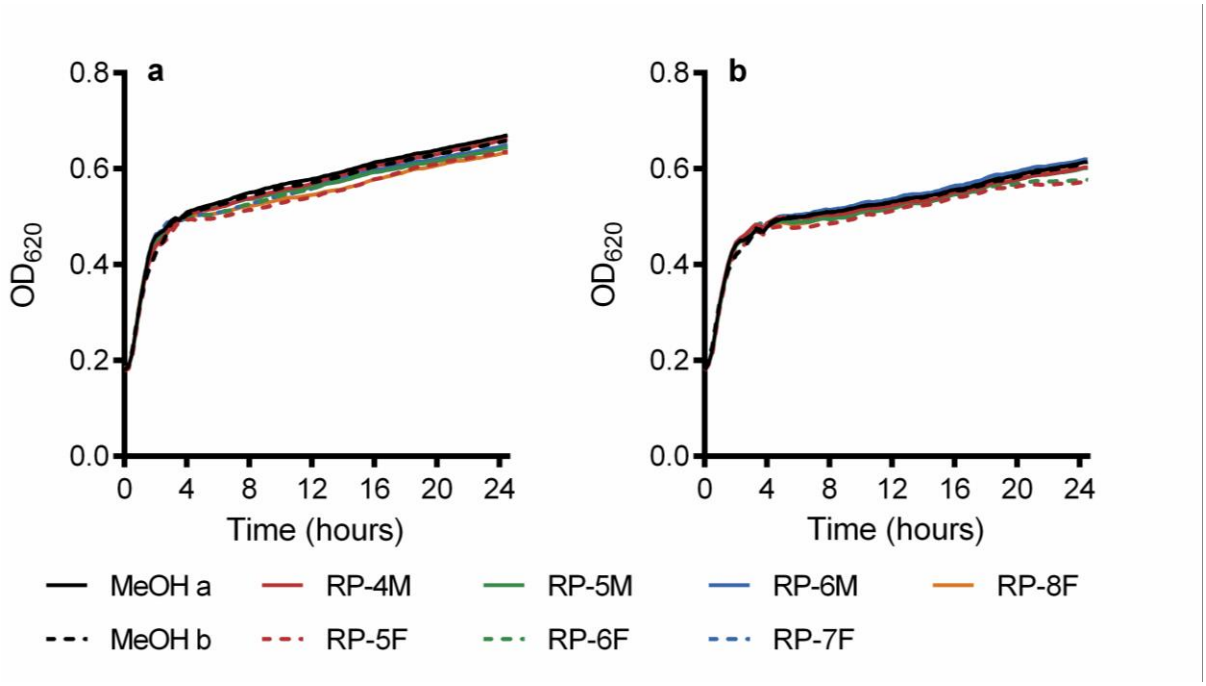
Appendix 25. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Finca los Nacientes *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



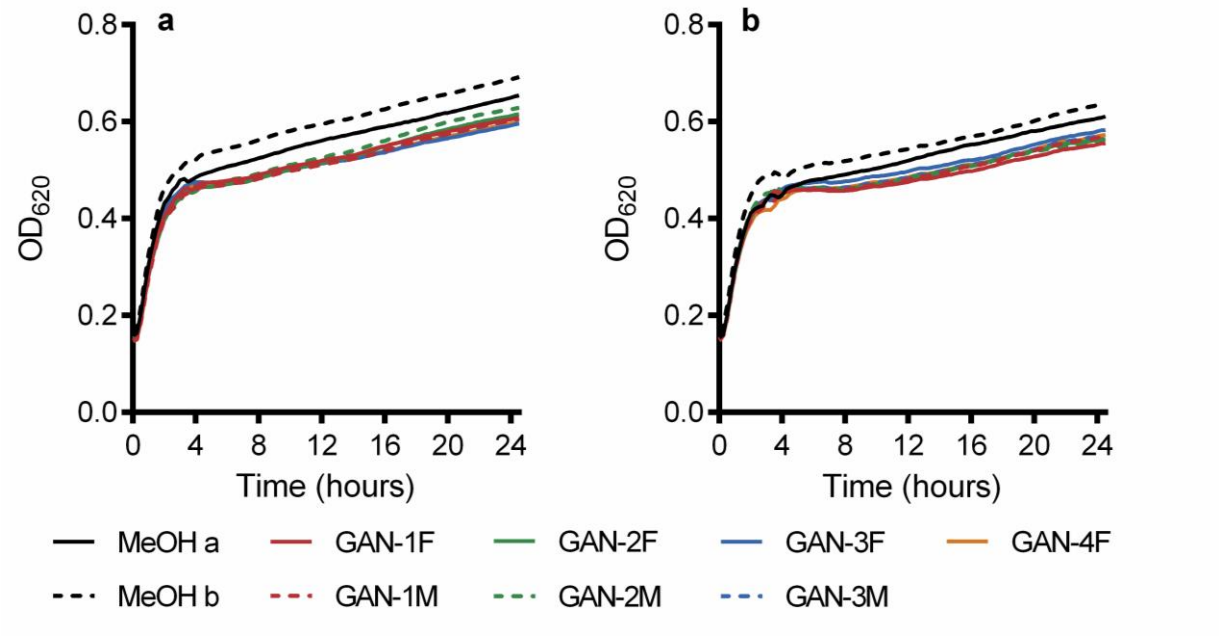
Appendix 26. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Finca los Nacientes *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.



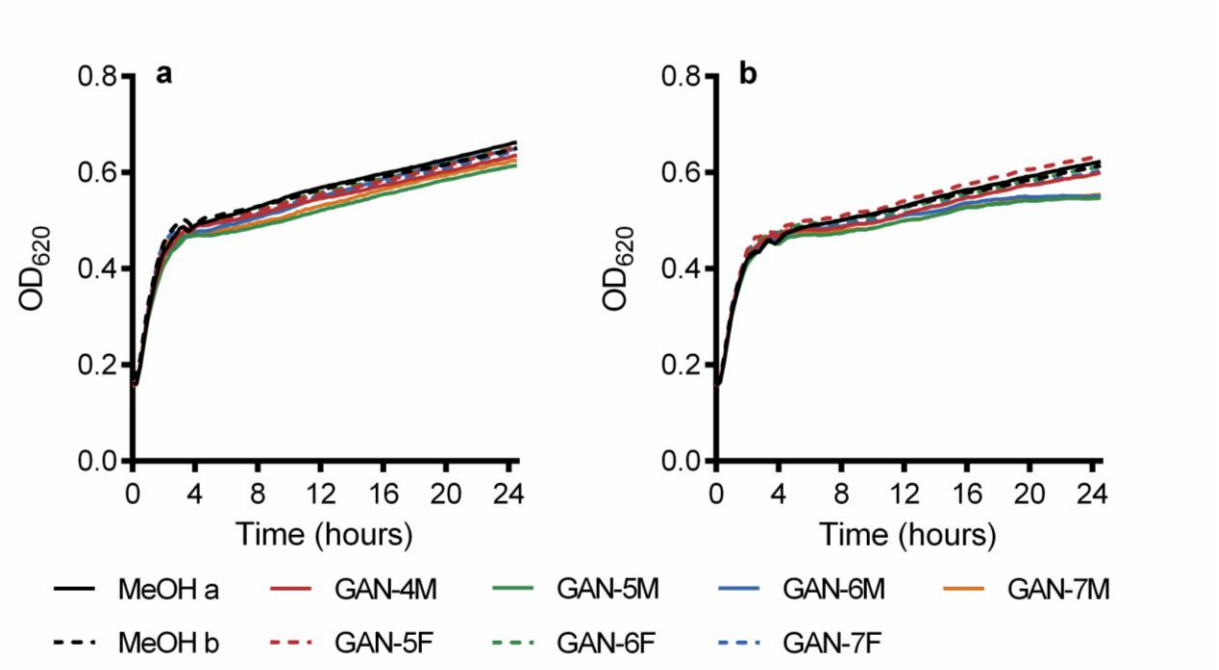
Appendix 27. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Río Palmas *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours



Appendix 28. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Río Palmas *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.

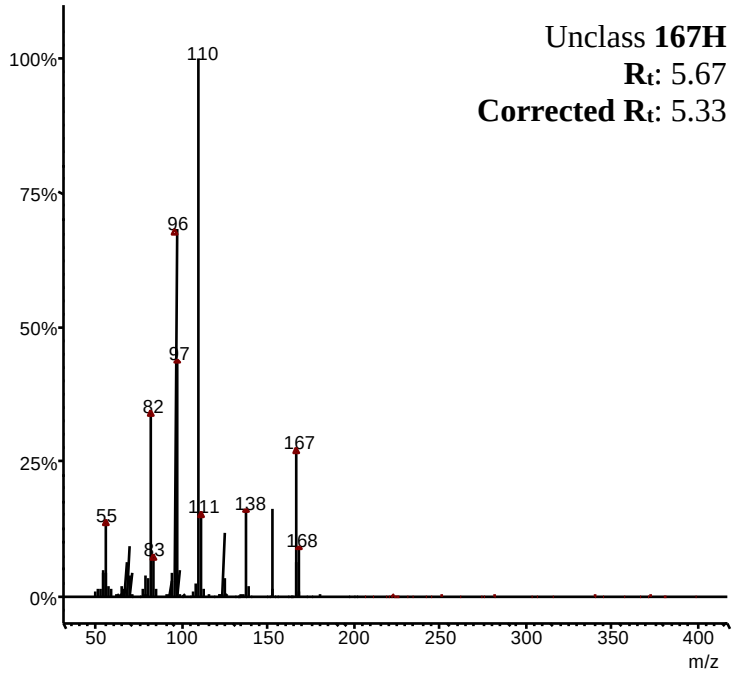


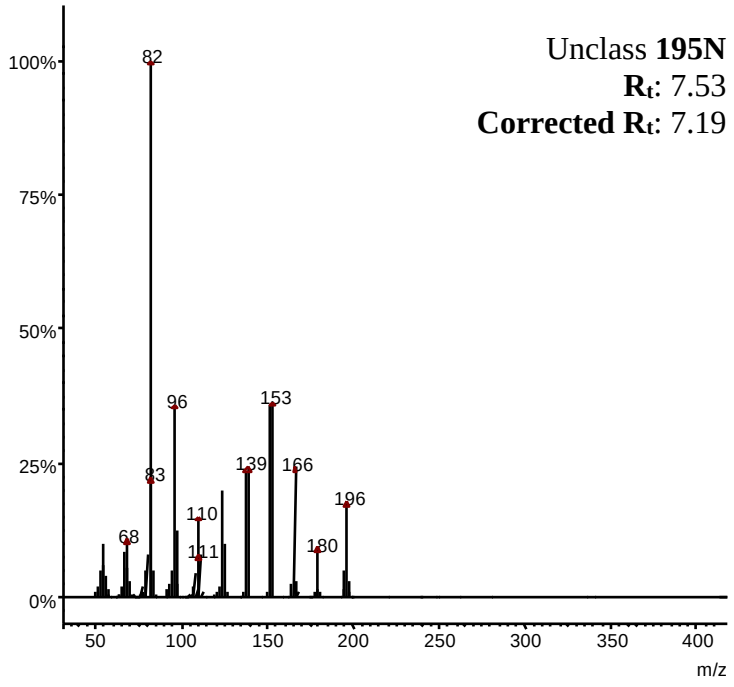
Appendix 29. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Gandoca *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.

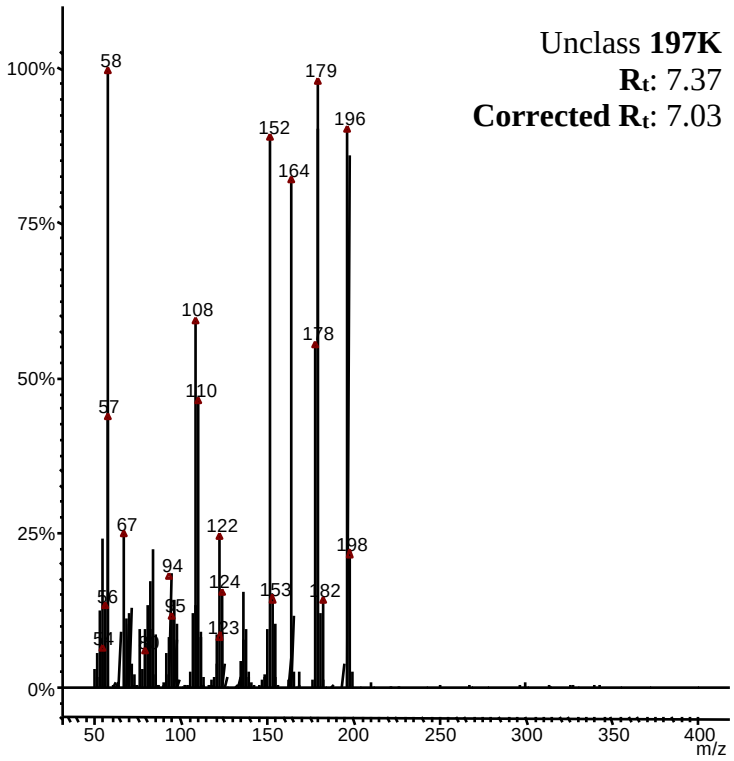


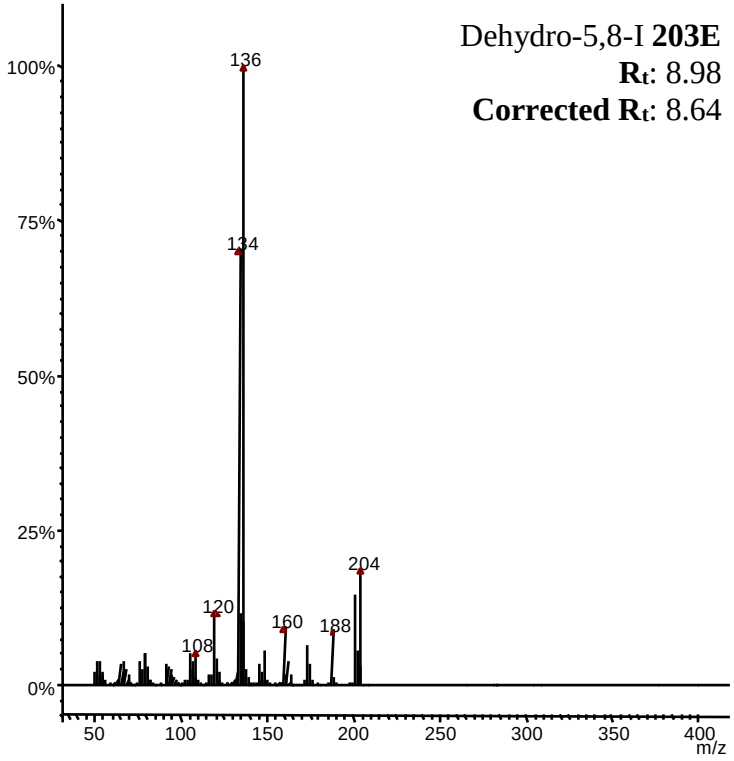
Appendix 30. Growth curves of *Klebsiella pneumoniae* treated with (**a**) 2.4 µL and (**b**) 4.8 µL methanol or Gandoca *Oophaga pumilio* alkaloids. OD_{620} measurements were recorded at 15-minute intervals over the course of 24.5 hours.

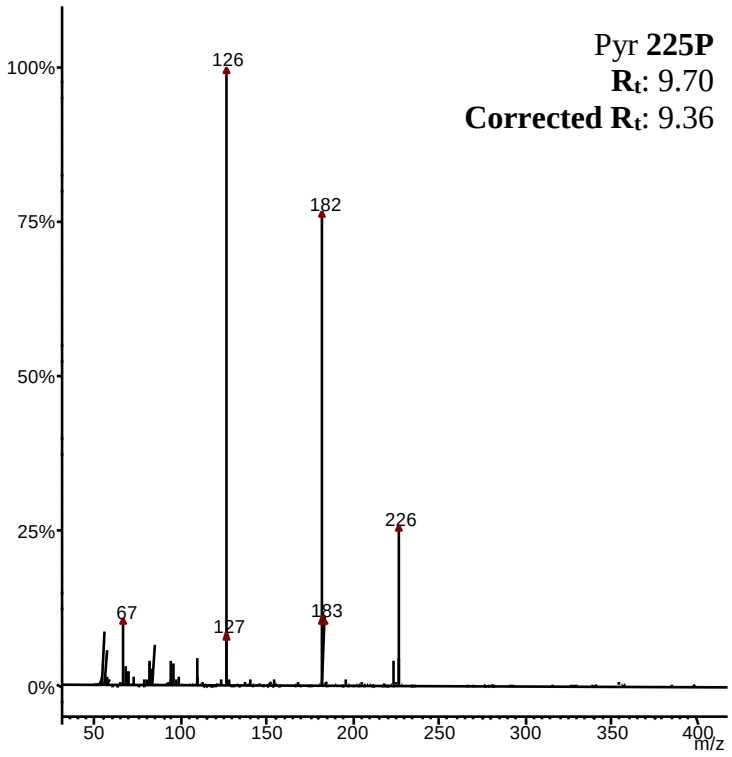
Appendix 31. Mass spectral data for the 27 new alkaloids detected in *Oophaga pumilio* (**Table 3**). The R_t for each alkaloid is included, along with an approximate "corrected R_t" to match the retention times in the alkaloid library of Daly *et al.* (2005). Following the methods of Garraffo *et al*. (2012), based on comparisons of R_ts for previously identified alkaloids in the present study with R_ts from Daly *et al*. (2005), alkaloids in the present study eluted approximately 0.34 seconds faster than times listed in Daly *et al*. (2005).

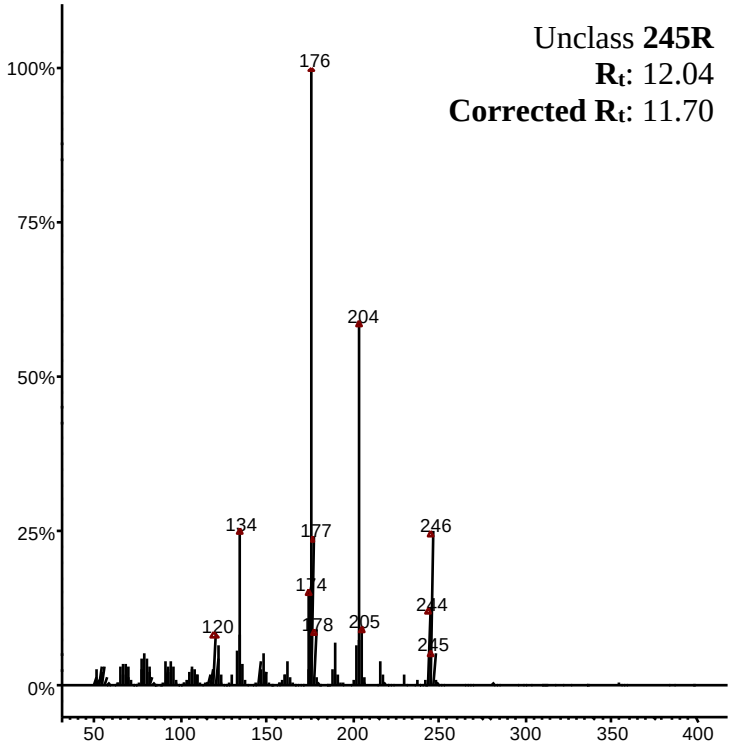


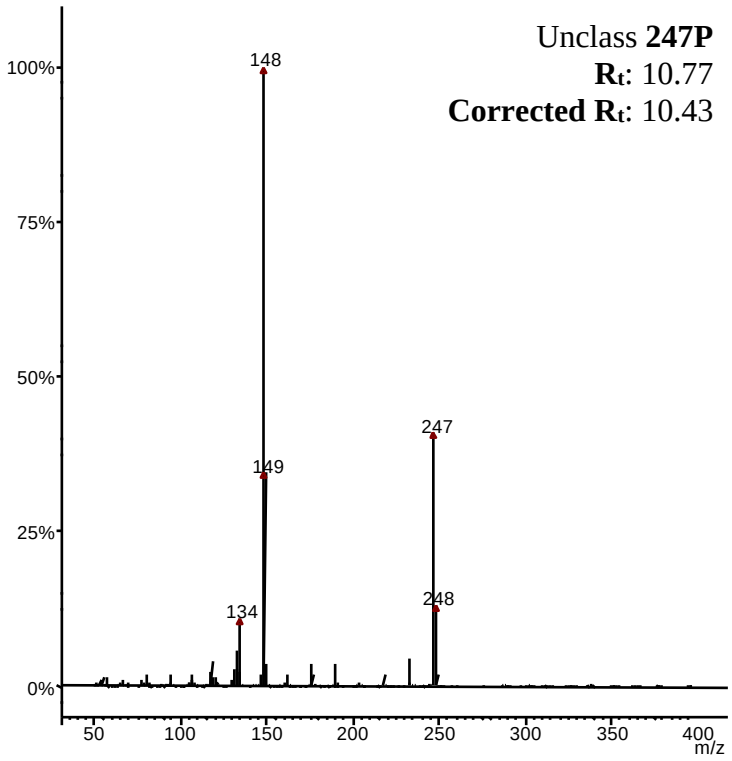


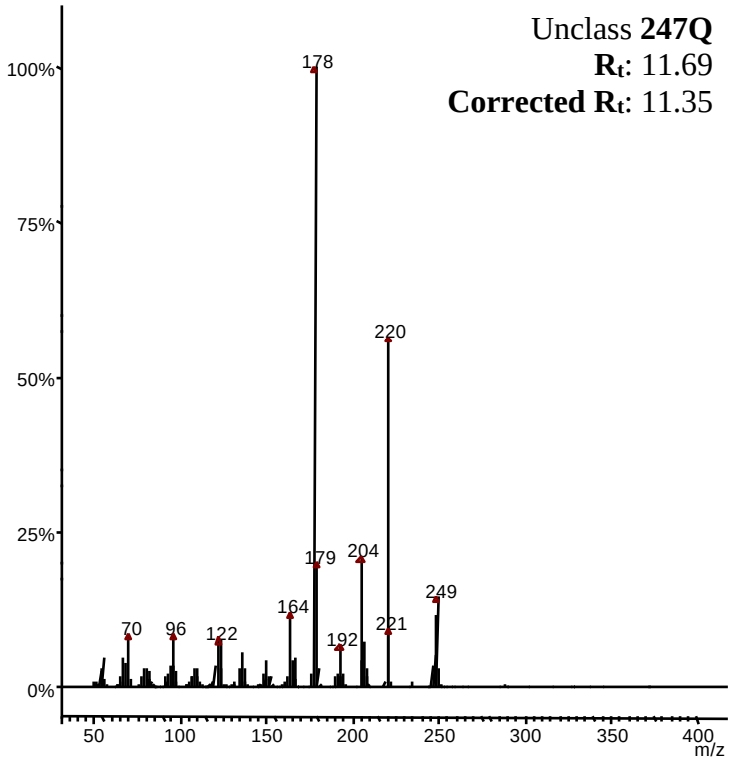

Unclass **197K**
Rt: 7.37
Corrected Rt: 7.03
100%
75%
50%
25%
0%
58
179
196
152
164
108
178
110
57
67
122
198
94
124
153
182
56
95
123
50
100
150
200
250
300
350
400
m/z

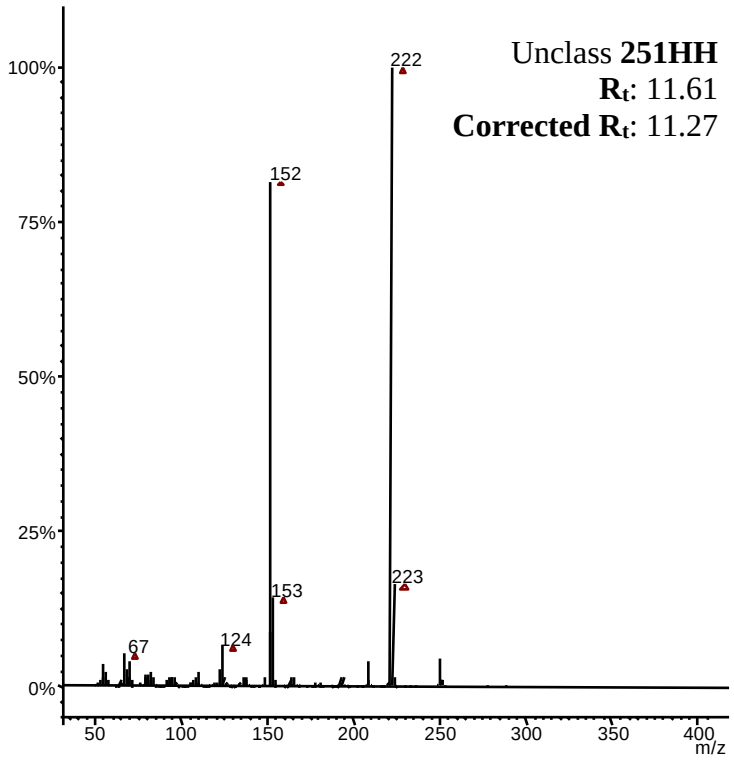

Dehydro-5,8-I **203E**
Rt: 8.98
Corrected Rt: 8.64
100%
75%
50%
25%
0%
136
134
204
120
160
188
108
50
100
150
200
250
300
350
400
m/z

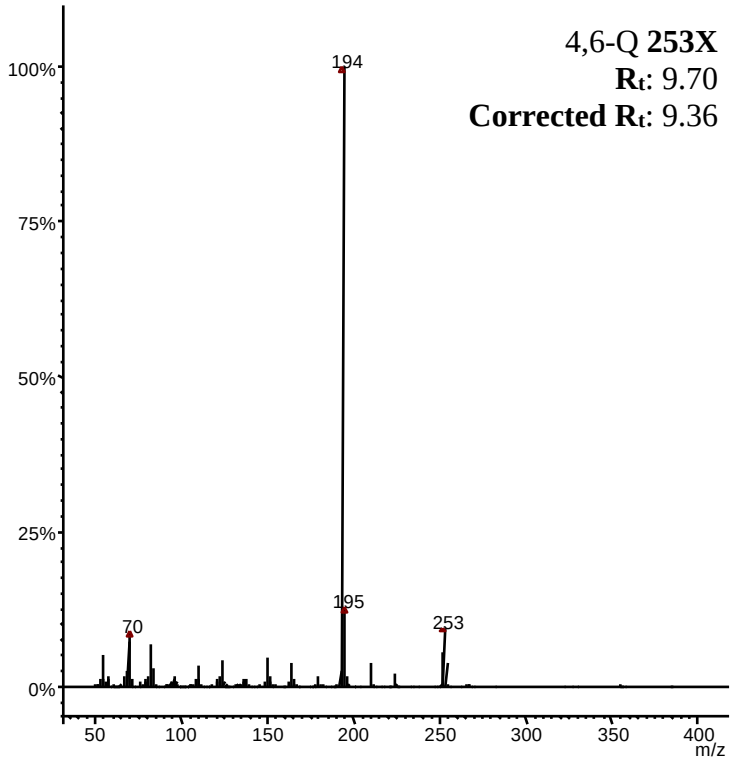

Pyr **225P**
R_t: 9.70
Corrected R_t: 9.36
100%
75%
50%
25%
0%
126
182
226
67
127
183
50
100
150
200
250
300
350
400
m/z

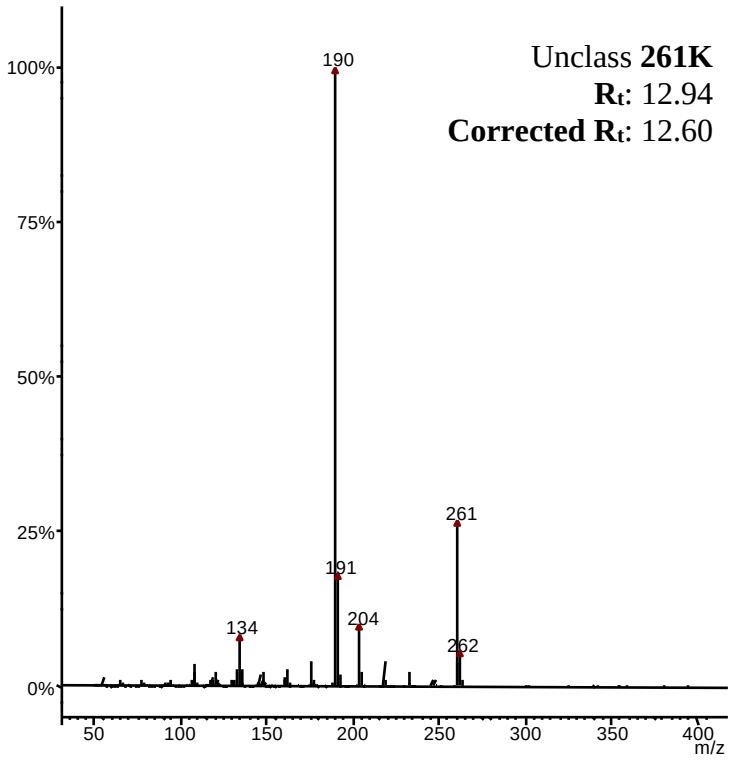

Unclass **245R**
R_t: 12.04
Corrected R_t: 11.70
100%
75%
50%
25%
0%
176
204
134
177
246
174
244
120
178
205
245
50
100
150
200
250
300
350
400

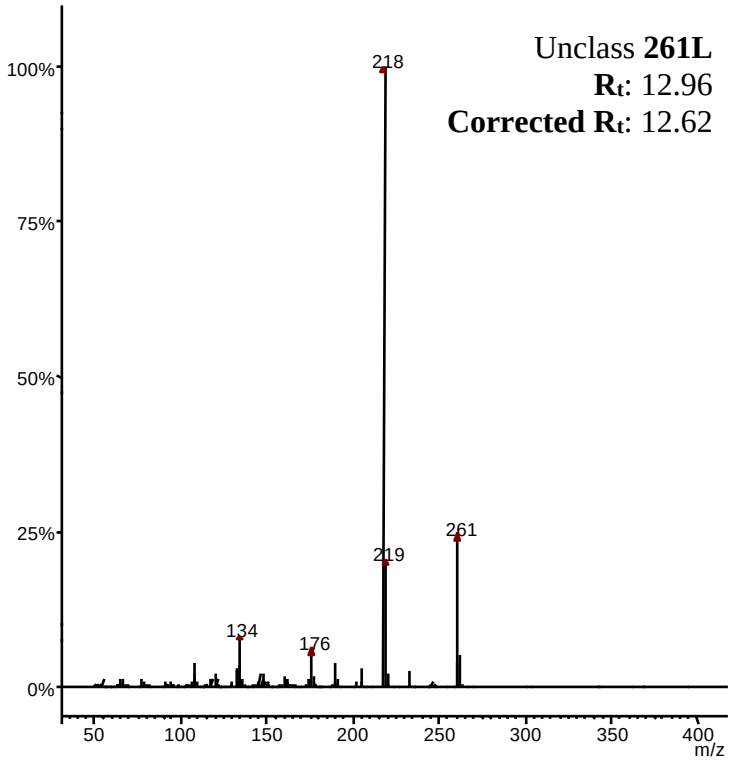

Unclass **247P**
Rt: 10.77
Corrected Rt: 10.43
100%
75%
50%
25%
0%
148
247
149
248
134
50
100
150
200
250
300
350
400
m/z

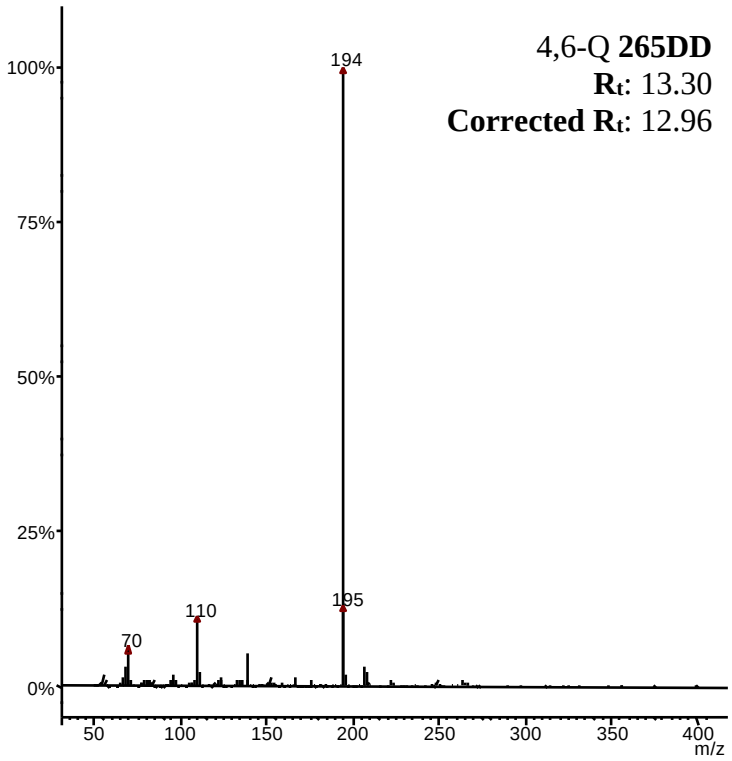

Unclass **247Q**
Rt: 11.69
Corrected Rt: 11.35
100%
75%
50%
25%
0%
178
220
179
204
249
164
70
96
122
192
221
50
100
150
200
250
300
350
400
m/z

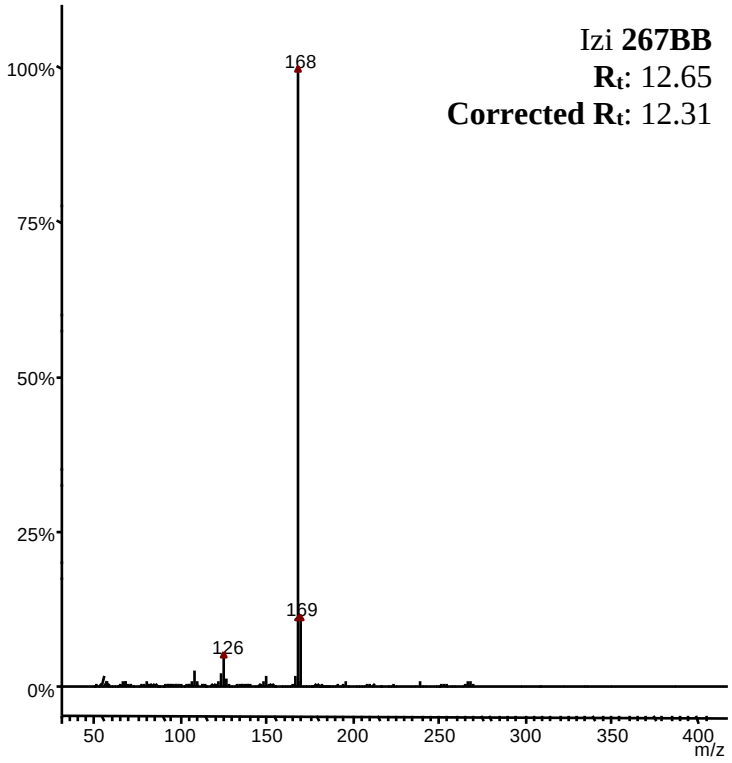

Unclass **251HH**
R_t: 11.61
Corrected R_t: 11.27
100%
75%
50%
25%
0%
222
152
153
223
67
124
50
100
150
200
250
300
350
400
m/z

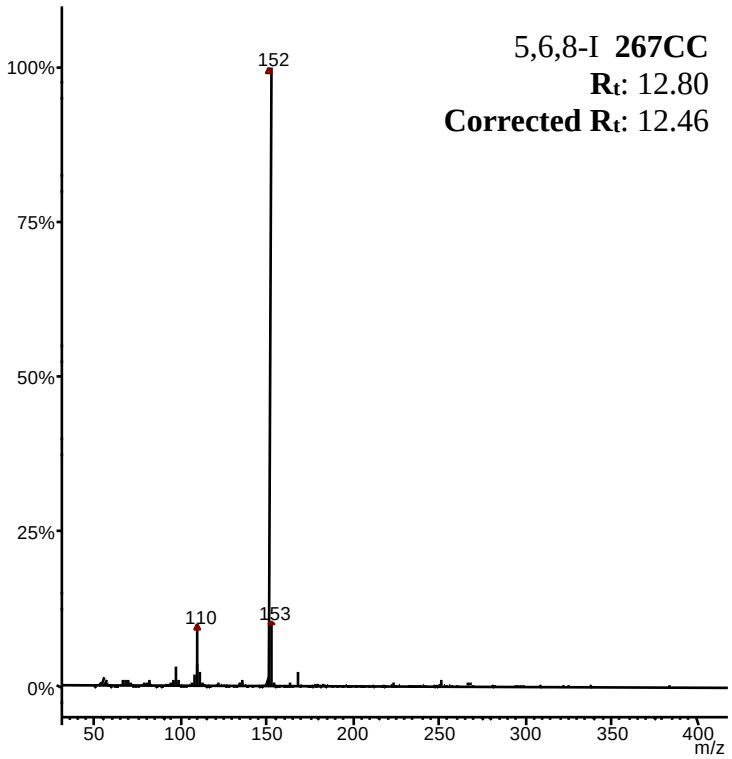

4,6-Q **253X**
R_t: 9.70
Corrected R_t: 9.36
100%
75%
50%
25%
0%
194
195
70
253
50
100
150
200
250
300
350
400
m/z

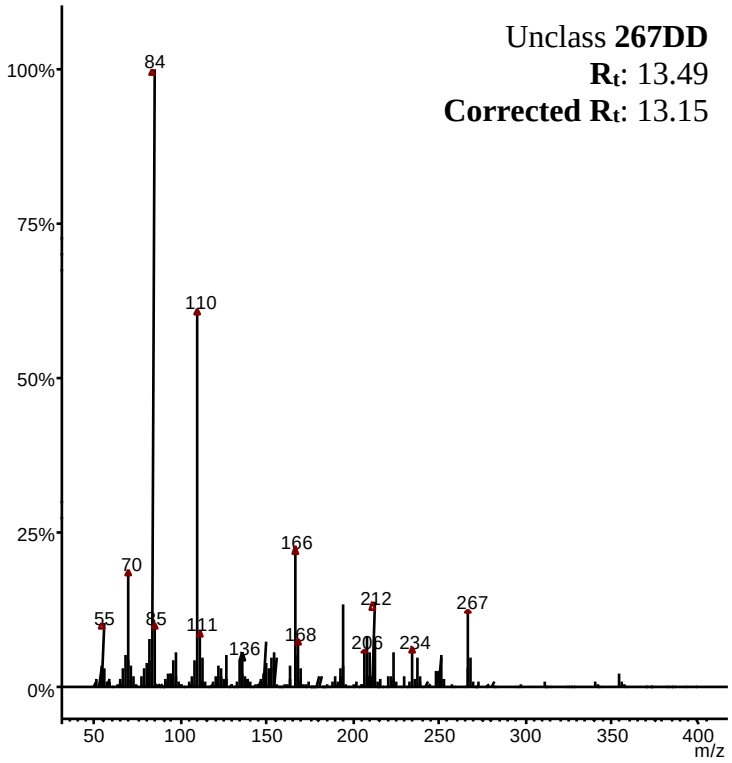

Unclass **261K**
Rt: 12.94
Corrected Rt: 12.60
100%
75%
50%
25%
0%
190
261
191
204
134
262
50
100
150
200
250
300
350
400
m/z

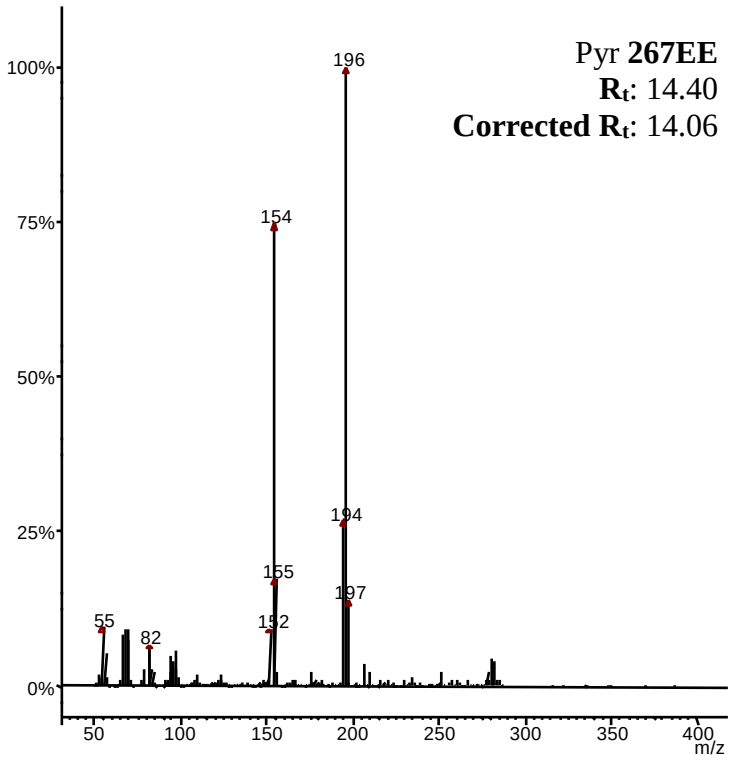

Unclass **261L**
Rt: 12.96
Corrected Rt: 12.62
100%
75%
50%
25%
0%
218
261
219
134
176
50
100
150
200
250
300
350
400
m/z

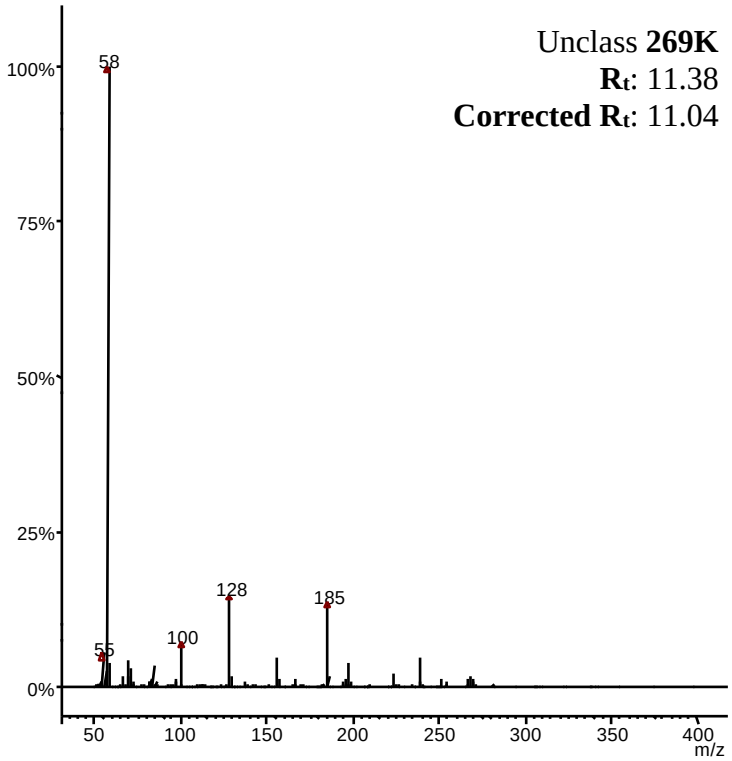

4,6-Q **265DD**
Rt: 13.30
Corrected Rt: 12.96
100%
75%
50%
25%
0%
194
195
110
70
50
100
150
200
250
300
350
400
m/z

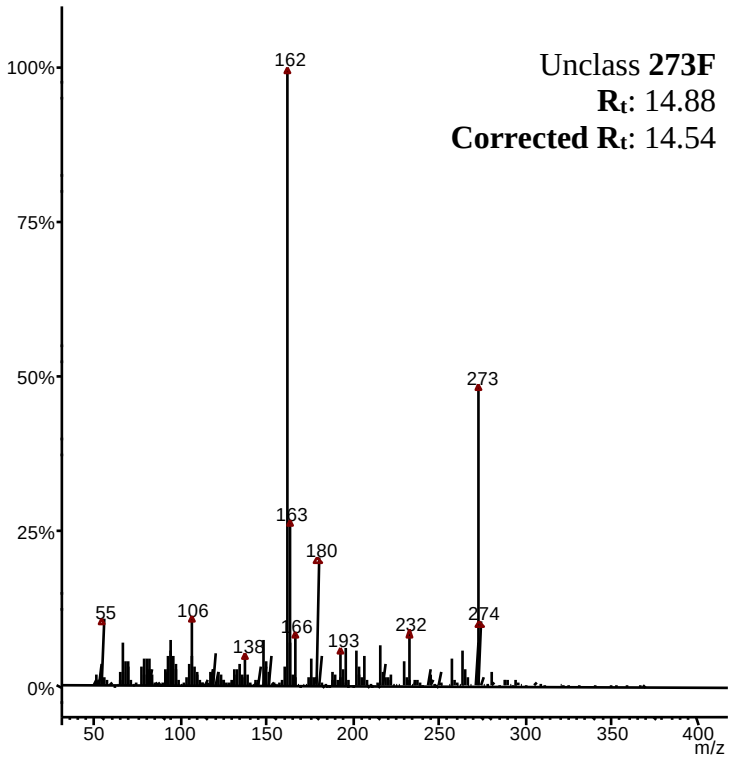

Izi **267BB**
Rt: 12.65
Corrected Rt: 12.31
100%
75%
50%
25%
0%
168
169
126
50
100
150
200
250
300
350
400
m/z

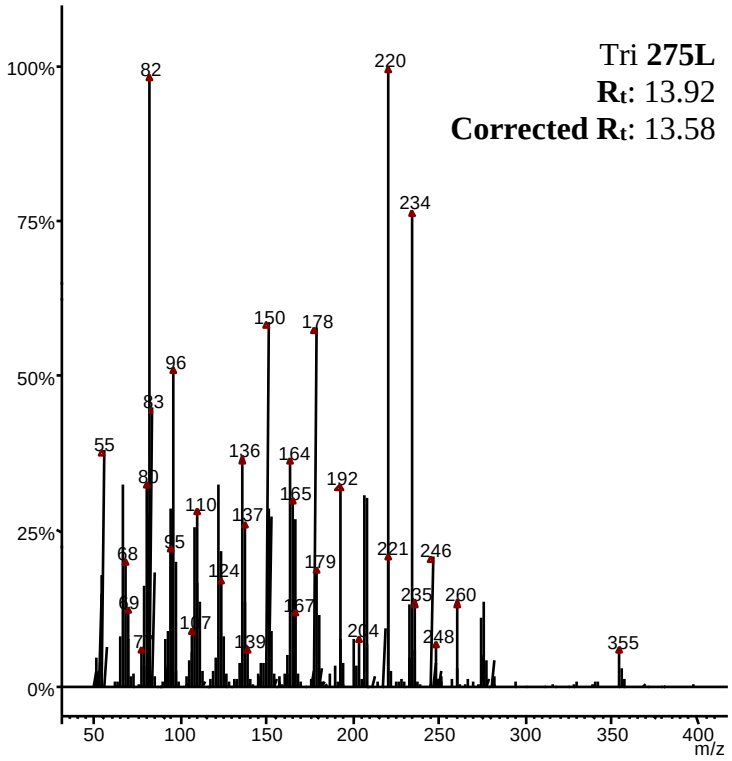

5,6,8-I **267CC**
Rt: 12.80
Corrected Rt: 12.46
100%
75%
50%
25%
0%
152
110
153
50
100
150
200
250
300
350
400
m/z

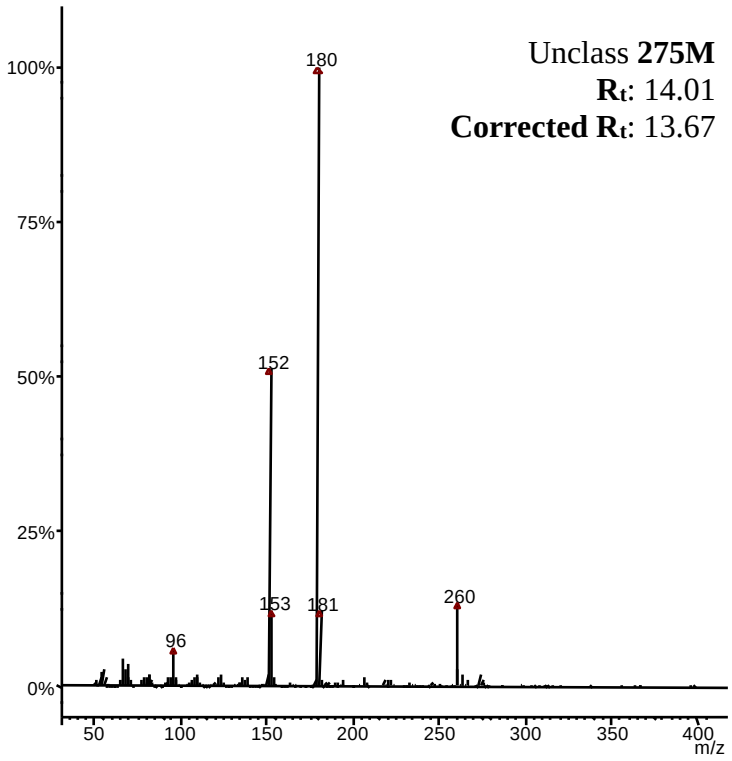

Unclass **267DD**
Rt: 13.49
Corrected Rt: 13.15
100%
75%
50%
25%
0%
84
110
166
70
212
267
55
85
111
168
136
206
234
50
100
150
200
250
300
350
400
m/z

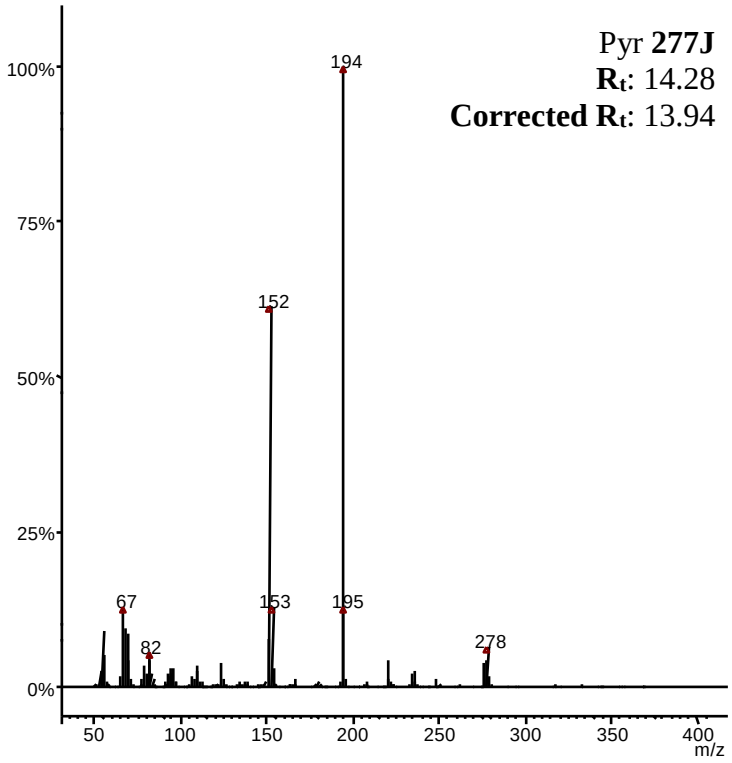

Pyr **267EE**
R_t: 14.40
Corrected R_t: 14.06
100%
75%
50%
25%
0%
196
154
194
155
197
55
82
152
50
100
150
200
250
300
350
400
m/z

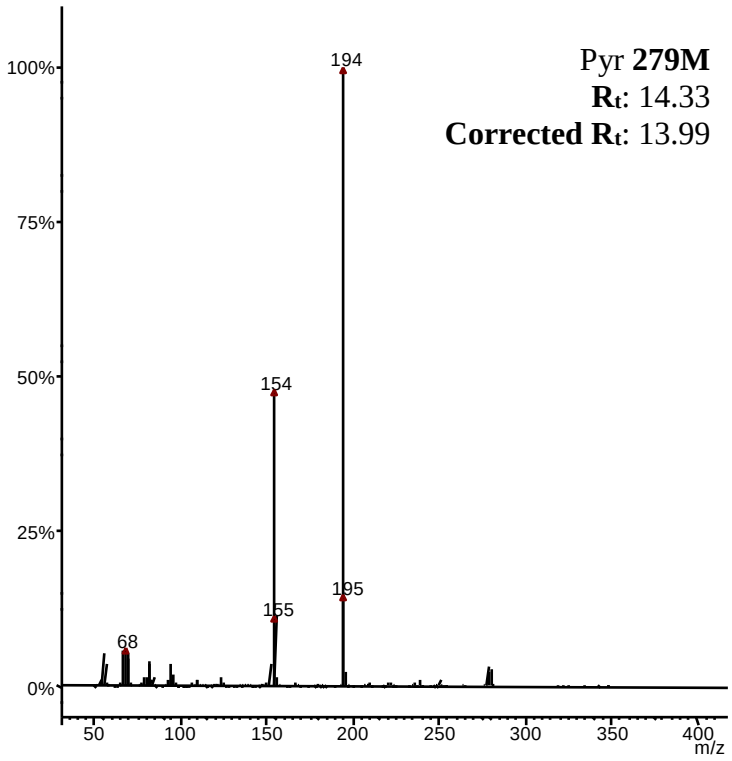

Unclass **269K**
R_t: 11.38
Corrected R_t: 11.04
100%
75%
50%
25%
0%
58
128
185
100
55
50
100
150
200
250
300
350
400
m/z

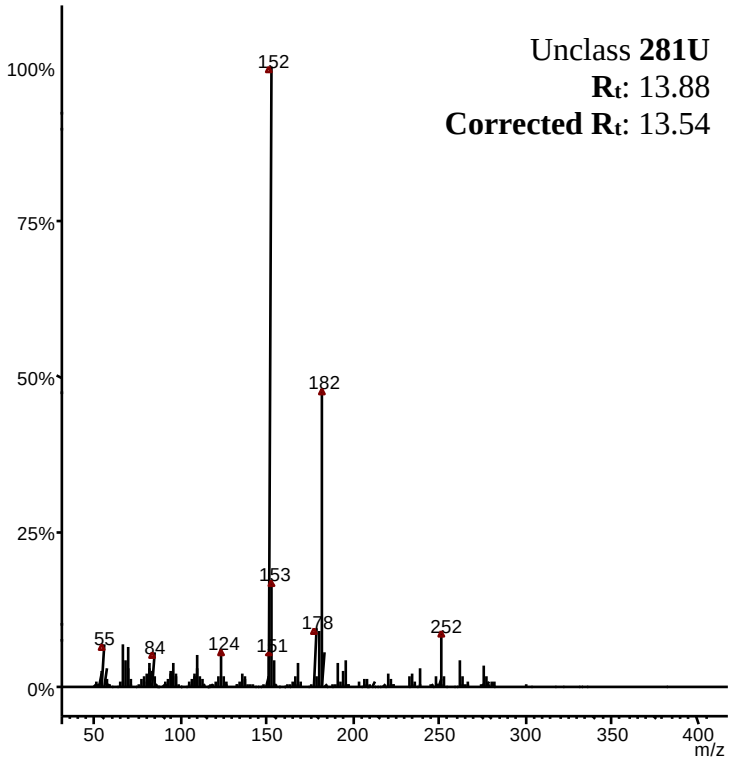

Unclass **273F**
R_t: 14.88
Corrected R_t: 14.54
100%
75%
50%
25%
0%
50
100
150
200
250
300
350
400
m/z
162
273
163
180
55
106
166
232
274
138
193

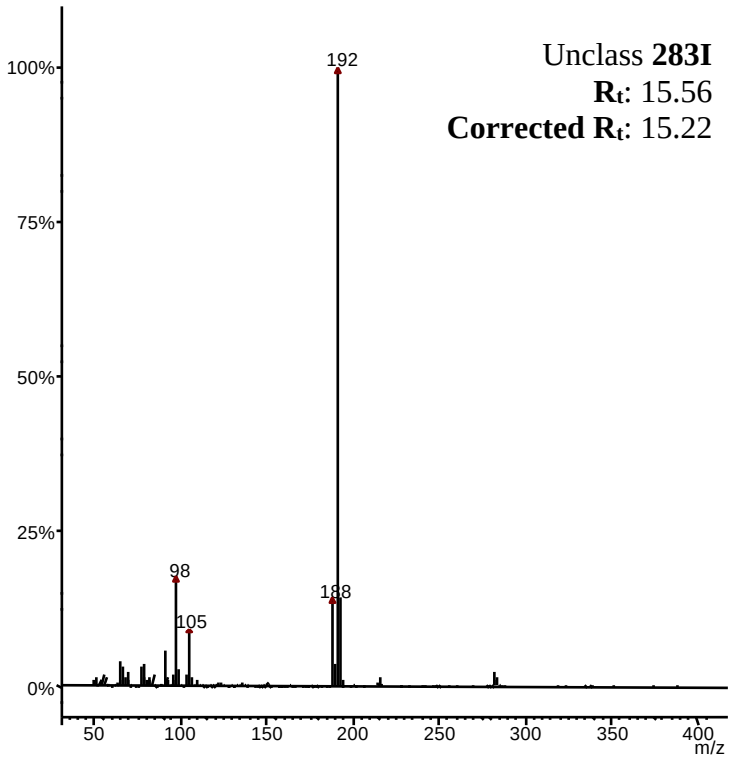

Tri **275L**
R_t: 13.92
Corrected R_t: 13.58
100%
75%
50%
25%
0%
50
100
150
200
250
300
350
400
m/z
82
220
234
150
178
96
83
55
136
164
80
192
165
110
137
95
68
124
221
246
179
69
235
260
167
107
204
248
355
71
139

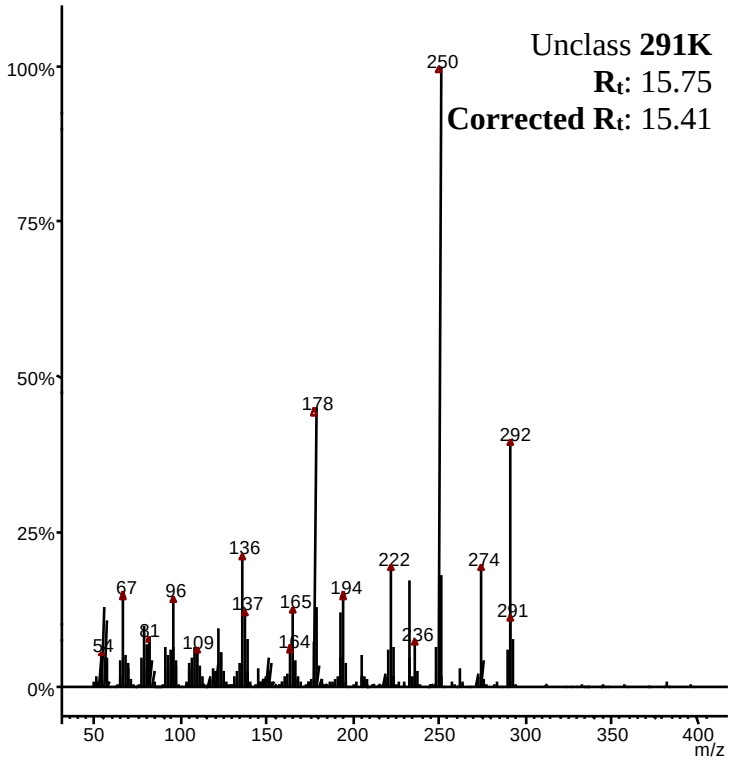

Unclass **275M**
Rt: 14.01
Corrected Rt: 13.67
100%
75%
50%
25%
0%
180
152
153
181
260
96
50
100
150
200
250
300
350
400
m/z

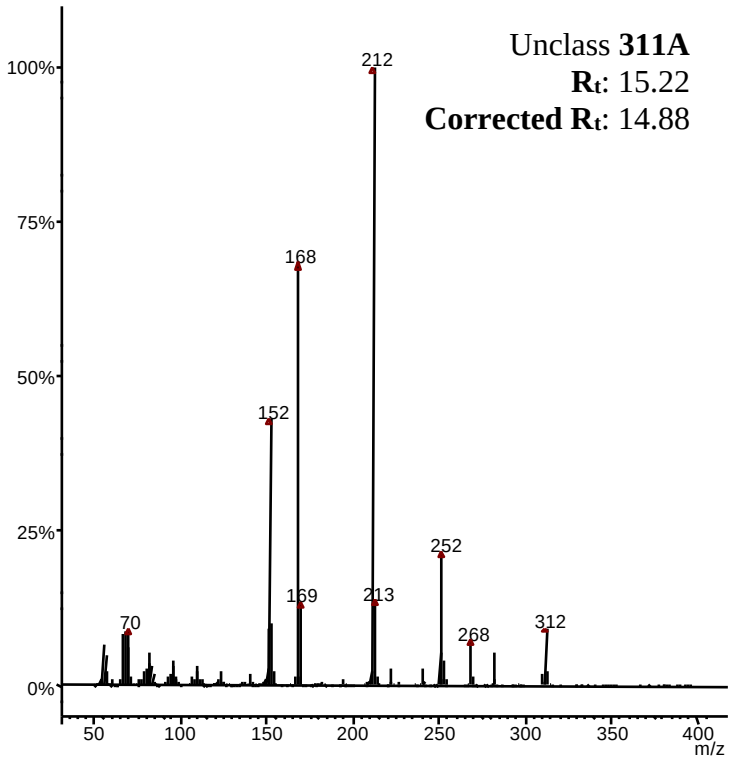

Pyr **277J**
Rt: 14.28
Corrected Rt: 13.94
100%
75%
50%
25%
0%
194
152
67
153
195
82
278
50
100
150
200
250
300
350
400
m/z


Pyr **279M**
Rt: 14.33
Corrected Rt: 13.99
100%
75%
50%
25%
0%
194
154
195
155
68
50
100
150
200
250
300
350
400
m/z


Unclass **281U**
Rt: 13.88
Corrected Rt: 13.54
100%
75%
50%
25%
0%
152
182
153
178
252
55
84
124
151
50
100
150
200
250
300
350
400
m/z


Unclass **283I**
Rt: 15.56
Corrected Rt: 15.22
100%
75%
50%
25%
0%
192
98
188
105
50
100
150
200
250
300
350
400
m/z


Unclass **291K**
Rt: 15.75
Corrected Rt: 15.41
100%
75%
50%
25%
0%
250
178
292
136
222
274
67
96
194
137
165
291
81
236
109
164
54
50
100
150
200
250
300
350
400
m/z


Unclass **311A**
R_t: 15.22
Corrected R_t: 14.88
100%
75%
50%
25%
0%
212
168
152
252
169
213
70
268
312
50
100
150
200
250
300
350
400
m/z